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Global Regulator of Virulence A (GrvA) Coordinates Expression of Discrete Pathogenic Mechanisms in Enterohemorrhagic *Escherichia coli* through Interactions with GadW-GadE

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ABSTRACT

Global regulator of virulence A (GrvA) is a ToxR-family transcriptional regulator that activates locus of enterocyte effacement (LEE)-dependent adherence in enterohemorrhagic *Escherichia coli* (EHEC). LEE activation by GrvA requires the Rcs phosphorelay response regulator RcsB and is sensitive to physiologically relevant concentrations of bicarbonate, a known stimulant of virulence systems in intestinal pathogens. This study determines the genomic scale of GrvA-dependent regulation and uncovers details of the molecular mechanism underlying GrvA-dependent regulation of pathogenic mechanisms in EHEC. In a *grvA*-null background of EHEC strain TW14359, RNA sequencing analysis revealed the altered expression of over 700 genes, including the downregulation of LEE- and non-LEE-encoded effectors and the upregulation of genes for glutamate-dependent acid resistance (GDAR). Upregulation of GDAR genes corresponded with a marked increase in acid resistance. GrvA-dependent regulation of GDAR and the LEE required *gadE*, the central activator of GDAR genes and a direct repressor of the LEE. Control of *gadE* by GrvA was further determined to occur through downregulation of the *gadE* activator GadW. This interaction of GrvA with GadW-GadE represses the acid resistance phenotype, while it concomitantly activates the LEE-dependent adherence and secretion of immune subversion effectors. The results of this study significantly broaden the scope of GrvA-dependent regulation and its role in EHEC pathogenesis.

IMPORTANCE

Enterohemorrhagic *Escherichia coli* (EHEC) is an intestinal human pathogen causing acute hemorrhagic colitis and life-threatening hemolytic-uremic syndrome. For successful transmission and gut colonization, EHEC relies on the glutamate-dependent acid resistance (GDAR) system and a type III secretion apparatus, encoded on the LEE pathogenicity island. This study investigates the mechanism whereby the DNA-binding regulator GrvA coordinates activation of the LEE with repression of GDAR. Investigating how these systems are regulated leads to an understanding of pathogenic behavior and novel strategies aimed at disease prevention and control.

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is a virulent intestinal pathogen causing foodborne outbreaks of bloody diarrhea (hemorrhagic colitis) and the life-threatening kidney disease hemolytic-uremic syndrome (1–3). Competitive colonization of the intestine by EHEC requires a type III secretion (T3S) mechanism and is characterized by the formation of attaching-and-effacing (A/E) lesions (1, 2). The T3S apparatus is encoded on a pathogenicity island, referred to as the locus of enterocyte effacement (LEE), containing 41 genes organized into five operons (*LEE1* to *LEE5*) (4). The master LEE regulator, Ler, encoded as the first gene of *LEE1*, positively stimulates LEE transcription by relieving H-NS-mediated repression. Ler further activates the LEE-encoded regulators GrlA and GrlR, which in turn activate and repress *ler* transcription, respectively (5–10). Numerous non-LEE-encoded regulators also converge on *ler* and *grlA* promoters to coordinate LEE-dependent colonization with environmental/physiological cues (11–19).

One such regulator, RcsB, has been shown to both activate and repress LEE transcription and more recently has been shown to be required for induction of the LEE in response to the bicarbonate ion (20, 21). RcsB is the response regulator of the Rcs phosphorelay system, a multicomponent signaling pathway including RcsC (sensor kinase), RcsD (histidine phosphotransferase), and RcsF (outer membrane lipoprotein) (22–26). Upon phosphorylation, RcsB can bind to target promoters as a homodimer and as a heterodimer in conjunction with other regulatory auxiliary proteins, such as RcsA, BglJ, and GadE (27–29). Binding occurs at specific RcsB consensus sites (30, 31), and the location of binding relative to the −35 consensus sequence can dictate whether RcsB positively or negatively affects transcription (32). Tobe et al. (21) demonstrated that upregulation of the LEE effector EspB in response to *rcsB* overexpression also required the transcriptional regulator GrvA (for global regulator of virulence A). Furthermore, the overexpression of *grvA* was shown to activate transcription from the *ler* promoter (*LEE1* operon) and to increase adherence to Caco-2 cells *in vitro*. In a subsequent study by Morgan et al. (20), both *rcsB* and *grvA* were shown to be required for bicarbonate induction of

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the LEE. Collectively, these two studies reveal GrvA to be an activator of Ler that can interact with RcsB to communicate the bicarbonate signal to the LEE colonizing mechanism.

How RcsB controls *grvA* and how GrvA, in turn, controls LEE expression are not known. GrvA shares homology with ToxR-family protein regulators, such as MarT in *Salmonella enterica* (30% amino acid identity) and CadC in *E. coli* (13% amino acid identity). Both MarT and CadC are transcriptional activators, with the latter activating the *cadBA* operon by displacing H-NS-mediated repression (33). The similarity of GrvA to MarT and CadC largely resides in the N terminus, containing a conserved DNA-binding helix-turn-helix domain (COG3710) and a CheY-like response regulator receiver domain (COG0745). In this study, details of the molecular basis for *grvA* regulation and for GrvA-dependent control of the LEE and LEE-dependent adherence are determined. Furthermore, global RNA sequencing analysis significantly broadens our knowledge of the role for GrvA in regulation to include genes central to acid resistance and metabolic fitness.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids used in this study are listed in Table 1. Strains were stocked at −80°C in glycerol diluted (final concentration, 15%, vol/vol) in Luria broth (LB) and were maintained in LB or on LB with 1.5% agar (LBA). Unless otherwise noted, overnight (18- to 20-h) cultures grown in LB were used to inoculate fresh LB or LB buffered with sodium bicarbonate (44 mM $NaHCO_3$) or fresh Dulbecco's modified Eagle's medium (DMEM; 4 g/liter glucose, 4 mM glutamine, 44 mM $NaHCO_3$, pH 7.4) to a final optical density at 600 nm (OD_{600}) of 0.05. Cultures were grown at 37°C in a rotary shaker (200 rpm) using a 1:10 medium-to-flask volume. Antibiotics (Sigma-Aldrich) were added to the cultures when required.

Genetic manipulations and complementation. Primers used for genetic manipulation and complementation are available upon request. For construction of deletion mutants, the bacteriophage λ Red-assisted one-step deletion method adapted for EHEC was used as described previously (20, 34, 35). Complementation of *rcsB* was performed using vector pRJM-20, as previously described (20). Additionally, a fragment containing the entire *rcsDB* operon, including the *rcsD* promoter region (nucleotide positions 3041665 to 3046191; GenBank accession number NC_013008.1), was cloned into BamHI/XbaI-digested low-copy-number expression vector pMPM-K3 (Kan^r) (36) using primers RcsB−3732/BamHI and RcsB+794/XbaI to produce pRJM-21. To complement *grvA*, a PCR product corresponding to the *grvA* open reading frame (ORF) and including a 267-bp upstream region of *grvA* containing the predicted promoter (nucleotide positions 1281984 to 1283134; GenBank accession number NC_013008.1) was produced using primers GrvA−268/XhoI and GrvA+884/BamHI and cloned into XhoI/BamHI-digested low-copy-number expression vector pMPM-A2 (Amp^r), creating pRJM-22. To complement *gadE*, a PCR product corresponding to the *gadE* ORF and 762 bp of the sequence upstream of *gadE* (containing the native promoters, nucleotide positions 4458857 to 4460195) was produced using primers GadE−763/XbaI and GadE+577/BamHI and cloned into the XbaI/BamHI-digested vector pMPM-A2 to create pRJM-30. Confirmation of genetic constructs was done using a combination of restriction mapping and DNA sequencing (MWG Operon).

RNA purification and qRT-PCR. The primers used for quantitative real-time PCR (qRT-PCR) are available upon request. RNA purification, cDNA synthesis, qRT-PCR cycling conditions, and data analysis followed previously described protocols (37) using a Realplex2 Mastercycler instrument (Eppendorf). Cycle threshold (C_T) data were normalized to the level of *rrsH* (16S rRNA gene) expression, and normalized cycle threshold (ΔC_T) values were transformed to arbitrary transcript expression levels using $2^{-\Delta CT}/10^{-6}$, as described previously (38, 39). Expression levels were compared statistically by the appropriate *t* test or by Tukey's honestly significant difference (HSD) test following a significant *F* test ($n \geq 3$, $\alpha = 0.05$; R software, version 3.1.0).

Construction of a single-copy *grvAB-luxE* operon fusion. A strain containing a single-copy chromosome-plasmid *luxE* reporter fusion was constructed using a protocol adapted from that of Shimizu et al. (40). To make a *grvAB-luxE* fusion, the *kan* cassette and flanking FLP recombination target sites were amplified from pKD4 (34) using primers pKD4forward/SacI and pKD4reverse/BamHI. This product was SacI/BamHI digested and cloned into the BamHI/SacI site of pMPM-T3 to produce pMPM-T3-*kan*. A XhoI/BamHI-digested PCR product containing the *luxE* gene and native ribosome binding site was amplified from p*laclux8* (40) using primers LuxE-18/XhoI and LuxE+1,450/BamHI and cloned into the XhoI/BamHI site of similarly digested pMPM-T3-*kan* to create pMPM-T3-*luxE-kan*. The *luxE-kan* PCR product amplified from pMPM-T3-*luxE-kan* using primers GrvA+1,283/LuxE and GrvA+1,431/P2 and Phusion high-fidelity DNA polymerase (NEB) was fused to a region 13 bp downstream of the *grvAB* operon using bacteriophage λ Red recombination (34). Kanamycin resistance cassettes were removed using FLP recombinase as described previously (34), and the *grvAB-luxE* construct was validated using a combination of restriction mapping and DNA sequencing (MWG Operon). The *lux* operon genes *luxCDAB* were constitutively expressed in *trans* in TW14359 *grvAB-luxE* from p*luxCDAB3* (40, 41).

Luciferase activity. Overnight cultures grown in LB and DMEM were used to inoculate fresh LB or DMEM, respectively, to an initial OD_{600} of 0.05. To measure the effect of sodium bicarbonate ($NaHCO_3$) on *grvA* expression, LB test cultures were grown with and without addition of 44 mM $NaHCO_3$ (all DMEM cultures contained 44 mM $NaHCO_3$). Cultures (0.2 ml) were inoculated into 96-well, clear-bottom white-walled plates (catalog number 655098; Greiner Bio-One) and incubated at 37°C in a rotary shaker (200 rpm). Luciferase and OD_{600} measurements were taken every hour for 10 h using a BioTek Synergy 2 plate reader (1 s integration) prewarmed to 37°C. Mean luciferase activity was compared between treatments (medium and $NaHCO_3$ effects) using the appropriate *t* test ($n = 4$, $\alpha = 0.05$; R software, version 3.1.0).

EMSAs. Electrophoretic mobility shift assays (EMSAs) were performed as previously described (42). Briefly, the forward primers RcsB+4/NcoI and RcsB+682/XhoI, used to PCR amplify *rcsB* from TW14359 DNA, contained a new start codon and an N-terminal 6×His epitope tag, and the PCR product was cloned into the NcoI/XhoI sites of similarly digested vector pET-24d to create pRJM-27. N-terminal 6×His-RcsB fusion proteins have wild-type RcsB activity (43). 6×His-RcsB was purified from strain BL21(DE3)pLysS using Ni-nitrilotriacetic acid spin columns (Qiagen) according to the manufacturer's protocol. Briefly, overnight LB cultures containing 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and kanamycin (50 μg/ml) grown at 18°C (200 rpm) were pelleted and resuspended in 1.5 ml of lysis buffer (10 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0) and lysed by sonication for 3 min (50% amplitude, 10-s intervals, 30-s pause) while they were chilled on ice. The lysate was centrifuged at 21,000 × *g* to remove insoluble cellular debris, and the supernatant was used for column purification and subsequently eluted with elution buffer (500 mM imidazole, 300 mM NaCl, 50 mM NaH_2PO_4, pH 8.0). Purified 6×His-RcsB protein was quantified using a Bradford protein assay. Five prime biotin-labeled primers (Integrated DNA Technologies oligonucleotides) were used to generate a probe containing the *grvA* promoter ($grvA_P$) region, including putative RcsB consensus binding sites (Frag-2F and Frag-2R) (Fig. 1B). An additional upstream *grvA* promoter probe with no predicted RcsB consensus sequence was amplified for use as a negative control (Frag-1F and Frag-1R) (Fig. 1B). Biotin-labeled probes were gel purified using QIAquick gel extraction kits (Qiagen) and diluted to a concentration of 0.5 ng/μl. All EMSAs were performed using a LightShift chemiluminescent EMSA kit (Thermo Pierce) according to the manufacturer's specifications. Prior to electrophoresis, purified 6×His-RcsB and biotin-labeled fragments were coincubated for 40 min in binding buffer (1× binding buffer, 50 ng/μl

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
DH5α	Vector propagation, *recA1 endA1*	
BL21(DE3)pLysS	BL21 with IPTG-inducible T7 polymerase	90
TW14359	Wild-type strain from the 2006 outbreak, western USA	91
EcRJM-1	TW14359 Δ*escN*	20
EcRJM-6	TW14359 Δ*rcsB*	20
EcRJM-10	TW14359 *rcsB*-FLAG	20
EcRJM-11	TW14359 Δ*grvA*	20
EcRJM-12	TW14359 Δ*rcsB* Δ*grvA*	20
EcRJM-13	TW14359 *tir*-FLAG	20
EcRJM-35	TW14359 Δ*grlR*::*kan*	20
EcRJM-72	TW14359 Δ*rcsB*/pRJM-20	20
EcRJM-73	TW14359 Δ*lacZ*	This study
EcRJM-74	TW14359/pRJM-23	This study
EcRJM-75	TW14359/pRJM-24	This study
EcRJM-76	TW14359/pRJM-25	This study
EcRJM-77	TW14359/pRJM-26	This study
EcRJM-78	TW14359 Δ*grvA*/pRJM-23	This study
EcRJM-79	TW14359 Δ*grvA*/pRJM-24	This study
EcRJM-80	TW14359 Δ*grvA*/pRJM-25	This study
EcRJM-81	TW14359 Δ*grvA*/pRJM-26	This study
EcRJM-82	TW14359 Δ*grvA* Δ*gadE*::*kan*/pRJM-24	This study
EcRJM-110	TW14359 Δ*grvA* Δ*gadE*::*kan*/pRJM-26	This study
EcRJM-83	TW14359 Δ*grvA*/pRJM-22	This study
EcRJM-84	TW14359 Δ*rcsB* Δ*grvA*/pRJM-22	This study
EcRJM-85	TW14359 Δ*rcsB* Δ*grvA*/pRJM-20	This study
EcRJM-86	TW14359 *rcsDB-luxE*	This study
EcRJM-87	TW14359 *grvAB-luxE*	This study
EcRJM-88	TW14359 *rcsDB-luxE*/p*luxCDAB3*	This study
EcRJM-89	TW14359 *grvAB-luxE*/p*luxCDAB3*	This study
EcRJM-90	TW14359 Δ*gadE*::*kan*	This study
EcRJM-91	TW14359 Δ*grvA* Δ*gadE*::*kan*	This study
EcRJM-92	TW14359 Δ*gadE*::*kan*/pRJM-30	This study
EcRJM-93	TW14359 Δ*grvA* Δ*gadE*::*kan*/pRJM-30	This study
EcRJM-94	TW14359 *zinT*-FLAG::*kan*	This study
EcRJM-95	TW14359 Δ*grvA zinT*-FLAG::*kan*	This study
EcRJM-96	TW14359/pRJM-31	This study
EcRJM-97	EPEC[a] E2348/69/pRJM-31	This study
EcRJM-98	MG1655/pRJM-31	This study
EcRJM-99	DH5α/pRJM-31	This study
EcRJM-100	TW14359/pRJM-32	This study
EcRJM-101	EPEC E2348/69/pRJM-32	This study
EcRJM-102	MG1655/pRJM-32	This study
EcRJM-103	DH5α/pRJM-32	This study
EcRJM-104	TW14359 *rcsDB-luxE*/p*luxCDAB3*/pRJM-21	This study
EcRJM-105	TW14359 *grvAB-luxE*/p*luxCDAB3*/pRJM-21	This study
EcRJM-106	TW14359 Δ*grvA*/pRJM-23	This study
EcRJM-107	TW14359 Δ*grvA*/pRJM-24	This study
EcRJM-108	TW14359 Δ*grvA*/pRJM-25	This study
EcRJM-109	TW14359 Δ*grvA*/pRJM-26	This study
Plasmids		
pACYC177	Low-copy-no. cloning vector, Amp[r] Kan[r] P15A	
pBAD22	Ara-inducible expression vector, Amp[r] M13	92
pMPM-A2	Low-copy-no. cloning vector, Amp[r] pMB1/f1	36
pMPM-K3	Low-copy-no. cloning vector, Kan[r] p15A/f1	36
pMPM-T3	Low-copy-no. cloning vector, Tet[r] p15A/f1	36
pUC19	High-copy-no. cloning vector, Amp[r] pMB1	93
pRS551	*lac* fusion vector, Amp[r] Kan[r] *lacZ*$^+$ ColE1	
pCP20	FLP recombinase expression vector	34
pKD4	Template plasmid for Kan cassette	34
pKM208	Bacteriophage λ Red recombinase expression vector	34

(Continued on following page)

TABLE 1 (Continued)

Strain or plasmid	Relevant characteristics	Reference or source
pET-24d	T7 promoter, His tag vector, Kan^r f1, pBR322	Novagen
pSU312	FLAG epitope template, Amp^r Kan^r, R6K	94
pRJM-20	pACYC177-*rcsB*	20
pRJM-33	pACYC177-*grvAB*	This study
pRJM-21	pMPM-K3-*rcsDB*	This study
pRJM-22	pMPM-A2-*grvA*	This study
pRJM-23	pRS551-$gadE_P$(−773, −1)[b]	This study
pRJM-24	pRS551-$gadE_P$(−320, −1)	This study
pRJM-25	pRS551-$gadE_P$(−516, −276)	This study
pRJM-26	pRS551-$gadE_P$(−773, −497)	This study
pRJM-27	pET-24d-6×His-*rcsB*	This study
pRJM-28	pMPM-T3-*kan*	This study
pRJM-29	pMPM-T3-*luxE-kan*	This study
pRJM-30	pMPM-A2-*gadE*	This study
pRJM-31	pRS551-$grvA_P$(−268, +38)	This study
pRJM-32	pUC-*grvA*-6×His	This study
p*laclux8*	p*laclux8*	40
p*LuxCDAB3*	p*LuxCDAB3*	40

[a] EPEC, enteropathogenic *E. coli*.
[b] The numbers indicate the positions in $gadE_P$.

sheared salmon sperm DNA, 2.5% glycerol, 0.05% NP-40, 5 mM $MgCl_2$, 50 mM KCl, 1 mM EDTA) at room temperature. Samples were loaded into a prerun and cooled 8% native polyacrylamide gel and run at 20 V/cm for 1 h. Following electrophoresis, biotin-labeled DNA was transferred (25 V for 20 min) to a Biodyne B precut modified nylon membrane (Thermo Pierce) and UV cross-linked for 60 s. Biotin-labeled DNA fragments were detected using streptavidin-horseradish peroxidase-conjugated antibodies (1:300) in blocking buffer, and the membranes were washed, equilibrated, and subsequently detected using luminol/enhancer solutions in a ChemiDoc XRS+ imaging system and Image Lab (version 3.0) software (Bio-Rad).

Construction of *lacZ* transcriptional fusions and β-galactosidase assays. Construction of a $grvA_P$-*lacZ* reporter transcriptional fusion followed a previously described protocol using pRS551 (Table 1) (20). Briefly, a 306-bp PCR product containing the predicted *grvA* promoter was amplified from TW14359 genomic DNA using primers GrvA−268/EcoRI and GrvA+38/BamHI, BamHI/EcoRI digested, and then cloned into similarly digested pRS551, resulting in pRJM-31. Four different $gadE_P$-*lacZ* promoter fusions were created by cloning BamHI/EcoRI-digested PCR products into similarly digested pRS551 using primers GadE−320/EcoRI and GadE−1/BamHI, GadE−276/EcoRI and GadE−516/BamHI, GadE−773/EcoRI and GadE−497/BamHI, and GadE−773/EcoRI and GadE−1/BamHI. β-Galactosidase activity (Miller units) was measured as previously described (38, 44), and the numbers of Miller units were compared using the appropriate *t* test or by Tukey's HSD test following a significant *F* test ($n \geq 3$, $\alpha = 0.05$; R software, version 3.1.0).

RNA sequencing and data analysis. RNA extractions were performed as described above for qRT-PCR, except that residual genomic DNA was digested using Turbo DNase (catalog number AM2238; Ambion) following the manufacturer's protocol. Three independent RNA samples for each strain (TW14359 and TW14359 Δ*grvA*) were pooled into a single sample ($n = 1$) in a 1:1:1 ratio to account for intersample variability, and rRNA was removed using a MICROBExpress bacterial mRNA enrichment kit (catalog number AM1905; Ambion) according to the manufacturer's instructions. The integrity and concentration of purified and enriched mRNA were determined using an Agilent 2100 bioanalyzer (Agilent Technologies). RNA sequencing (RNA-seq) was performed as previously described (45). Briefly, mRNA was converted to strand-specific cDNA using an Ion Total RNA-Seq kit (version 2; catalog number 4475936; Ion Torrent), and samples were sequenced using an Ion Personal Genome Machine (PGM) system (Ion Torrent). Sequence mapping and data analysis were performed using CLC Genomics Workbench (version 6) software (CLC Bio). Subsequent reads per kilobase per million mapped reads (RPKM) values were converted to the fold change for TW14359 Δ*grvA* relative to the value for wild-type strain TW14359. Circular plots were created using Circos (version 0.65) software (http://www.circos.ca) (46).

Intestinal cell adherence assays. The maintenance and culture of HT-29 colonic intestinal cells were performed as previously described (20). Adherence competition indexes were determined using the method of Gabbianelli et al. (47). Briefly, overnight DMEM cultures were used to inoculate fresh DMEM to an OD_{600} of 0.05, and these were then incubated for 3 h at 37°C with shaking (200 rpm). Cultures were then diluted to an OD_{600} of 0.5 and mixed in a 1:1 ratio (test strain/control strain), and 0.2 ml of each was used to inoculate HT-29 cells in 6-well culture plates. A control strain incapable of utilizing lactose (a Lac$^-$ strain) was constructed by deleting the entire *lacZ* ORF in TW14359 (strain EcRJM-73) using the bacteriophage λ Red approach and deletion primers LacZ−1/P1 and LacZ+3115/P2 (primer sequences are available upon request). Each plate was gently mixed before centrifugation at 500 × *g* for 5 min and then incubated as described above. Following 3 h of incubation, each well was washed four times using sterile phosphate-buffered saline (PBS) to remove nonadherent cells, and adherent cells were removed using 500 μl of 0.1% Triton X-100. Cells were enumerated (number of CFU per milliliter) by serial dilution in PBS and plating onto MacConkey agar, which is differential for lactose utilization. After overnight growth on MacConkey agar, pink (Lac-positive [Lac$^+$]) colonies were scored as the numbers of CFU per milliliter for the test strain, whereas white (Lac-negative [Lac$^-$]) colonies were scored as the numbers of CFU per milliliter for strain TW14359 Δ*lacZ*. The competitive index was derived by dividing the numbers of CFU per milliliter of the test strain by the numbers of CFU per milliliter of TW14359 Δ*lacZ*, and the indexes were compared statistically using the appropriate *t* test ($n \geq 3$, $\alpha = 0.05$; R software, version 3.1.0).

Test for acid resistance. Acid resistance by the glutamate-dependent system was measured as described previously (37) with slight adaptations. Mid-exponential-phase ($OD_{600} = 0.5$) DMEM cultures were inoculated to a 10^6-CFU/ml final cell density in E minimal glucose (EG) medium with or without 5.7 mM L-glutamate at pH 7 (control) or acidified with HCl (pH 2). For cell count (number of CFU per milliliter) and percent survival determinations, samples were serially diluted in PBS (pH 7), plated onto LBA, and incubated overnight at 37°C.

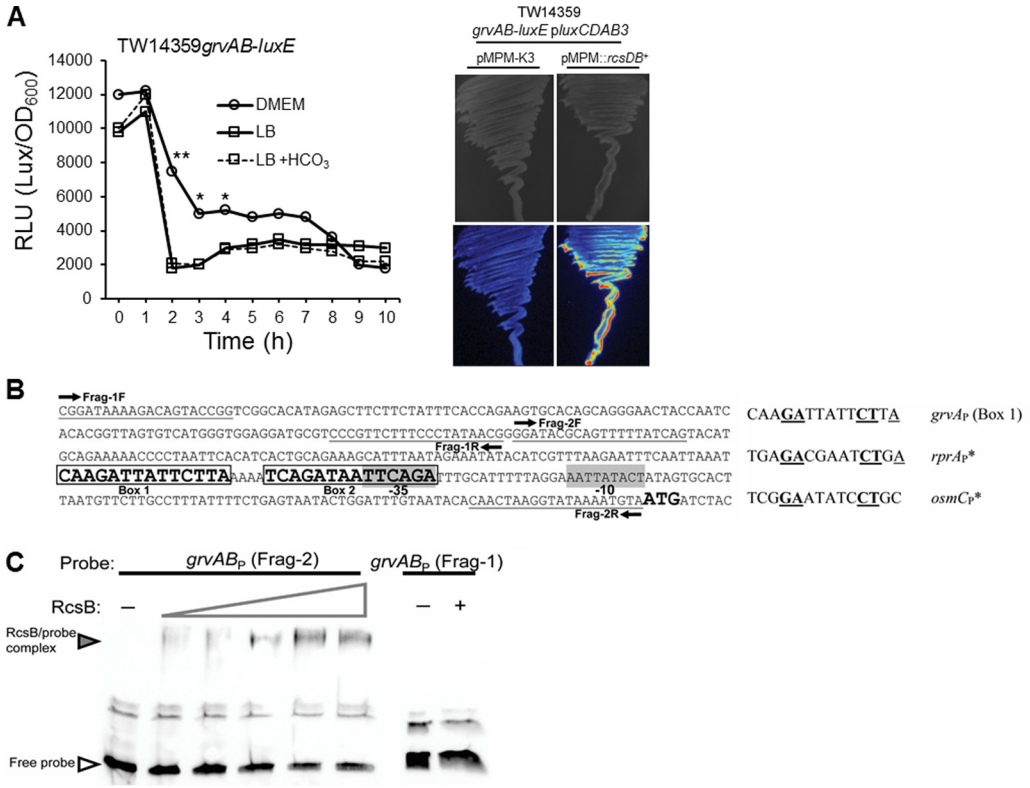

FIG 1 Rcs response regulator RcsB directly activates transcription from the *grvAB* promoter. (A) (Left) Average luciferase activity (in relative light units [RLU]) plotted as a function of time for a single-copy *grvAB-luxE* fusion grown in DMEM, LB, or LB supplemented with 44 mM $NaHCO_3$. Asterisks denote significance by Student's *t* test (*, $P < 0.05$; **, $P < 0.01$; $n \geq 3$). Plots at each time point did not vary from the mean by more than 5%. (Right) Growth and *grvAB* promoter expression (luciferase activity) (bottom) on LB agar plates for strain TW14359 *grvAB-luxE*/p*luxCDAB3* transformed with empty vector pMPM-K3 (left) or pMPM::*rcsDB* (right). (B) (Left) Map of the *grvAB* promoter and flanking regions in strain TW14359. Fragments for EMSA (Frag-1 and Frag-2) and the respective primers for probe generation are indicated. Frag-1 is the negative control; Frag-2 contains two putative RcsB binding sites (Box 1 and Box 2). (Right) Alignment of box 1 from the *grvAB* promoter with experimentally proven RcsB homodimer boxes for *rprA* and *osmC* promoters. Conserved bases are in bold and underlined. (C) EMSA for RcsB binding to Frag-2 of the *grvAB* promoter. The wedge denotes the increasing amount of RcsB added (1 to 12 μg). For Frag-1 (control), RcsB was added at 0 μg (lane −) and 12 μg (lane +).

RESULTS

Transcription of *grvA* is growth phase dependent and directly activated by RcsB. Previous work has shown the response regulator of the *rcs* phosphorelay system RcsB to be an activator of global regulator of virulence A (*grvA*) and both factors to be required for full bicarbonate induction of the LEE-encoded regulator Ler and subsequent stimulation of LEE expression in EHEC during exponential-phase growth (20, 21). RcsB is predicted to act upstream of *grvA* in the regulation of *ler*, yet the mechanism underlying the control of *grvA* by RcsB is unknown.

The level of transcription of *grvA* in reporter strain TW14359 *grvAB-luxE*/p*luxCDAB3* (EcRJM-89) (Table 1) grown in DMEM with sodium bicarbonate (44 mM) was the highest during early exponential-phase growth, rapidly declining as cultures transitioned into postexponential phase (Fig. 1A). When grown in LB, the level of *grvA* transcription was similar to that observed when grown in DMEM; however, the levels were consistently lower over the first 7 h. Addition of sodium bicarbonate (44 mM) to LB, previously shown to upregulate RcsB levels (20), had no significant impact on *grvA* transcription, while overexpression of *rcsDB* in *trans* in TW14359 *grvAB-luxE*/p*luxCDAB3* substantially increased the level of *grvA* transcription when grown on LB agar in the absence of sodium bicarbonate (Fig. 1A). Thus, the addition of bicarbonate alone is not sufficient for upregulation of *grvA*. That overexpression of *rcsB* increased the level of transcription from the *grvA* promoter suggests that RcsB may directly activate *grvA* transcription. In support of this, two putative tandem RcsB binding sites proximal to a predicted −35 site (nucleotides 1282968 to 1282982 in TW14359) of the *grvA* promoter ($grvA_P$) have been described previously (21, 31) (Fig. 1B, Box 1 and Box 2). To test for interactions between RcsB and the *grvA* promoter, purified 6×His-tagged RcsB was coincubated with a fragment of the core $grvAB_P$ element, including putative RcsB binding sites (Fig. 1B, Frag-2) and analyzed using electrophoretic mobility shift assays. As anticipated, the 235-bp $grvAB_P$ fragment was visibly shifted with the addition of increasing amounts (1 to 12 μg) of 6×His-RcsB, while no shift was observed for the control fragment (Fig. 1B, Frag-1, and C). These findings suggest that RcsB is a direct transcriptional activator of *grvA* and that activation occurs at the *grvA* core promoter region containing at least one putative RcsB binding site.

GrvA is a regulator of pathogenic mechanisms and metabolic fitness in EHEC. *grvA* is required for LEE activation by the Rcs phosphorelay system in EHEC (21). How, in turn, GrvA upregulates LEE expression and the scope of genes under the control of GrvA are unknown. To explore this, the transcriptomes of EHEC

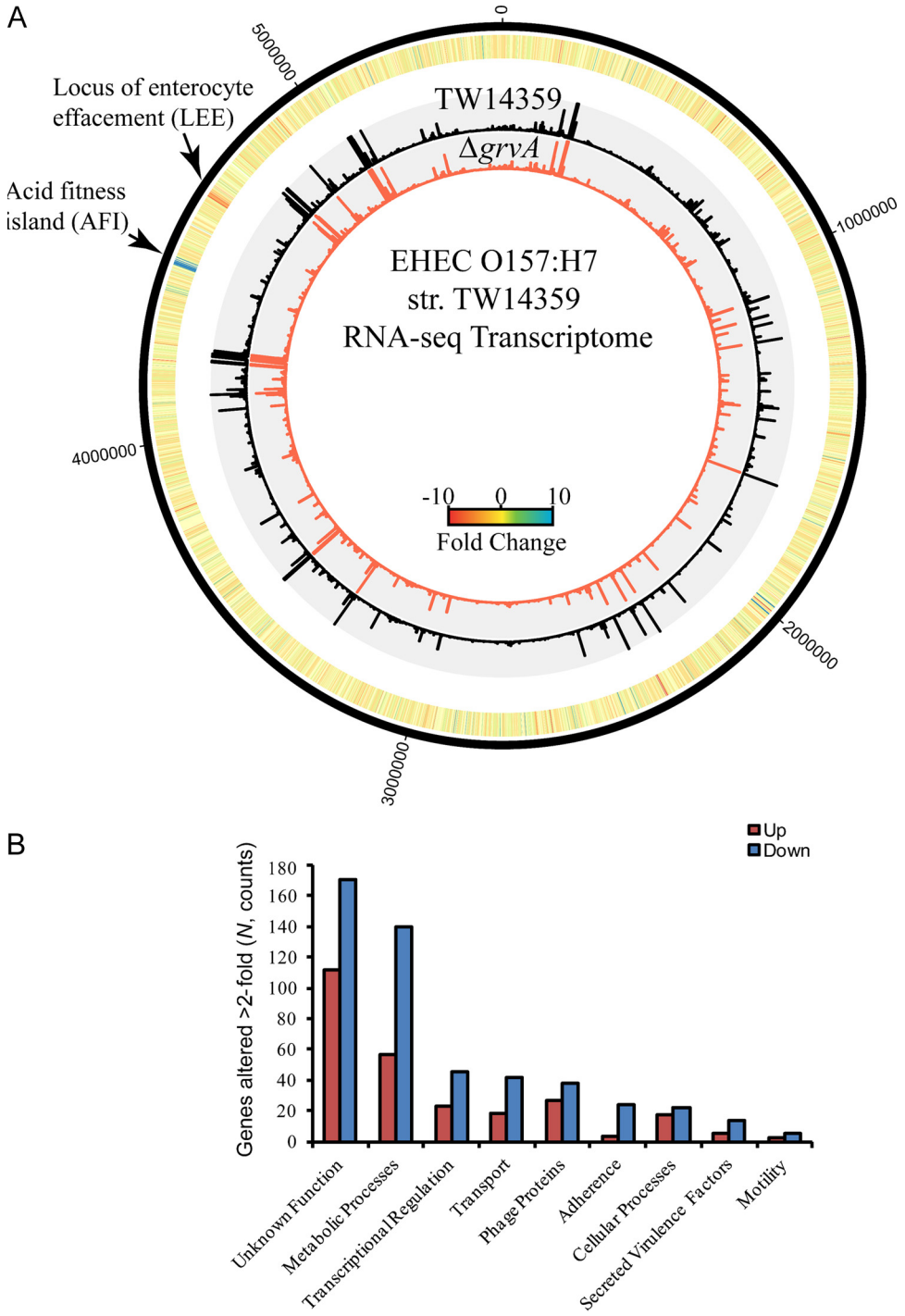

FIG 2 The GrvA regulon in EHEC strain TW14359. (A) Circular plot of RNA-seq data for EHEC strains TW14359 and TW14359 Δ*grvA*. Nucleotide positions are based on the TW14359 annotation (GenBank accession number NC_013008.1), indicated by the black outer ring. The heat map denotes the fold change in RPKM for TW14359 Δ*grvA* relative to the level of expression in TW14359 for each gene. The two inner histograms indicate the RPKM values of TW14359 (black) and TW14359 Δ*grvA* (red). Plots were generated using Circos (version 0.65; http://www.circos.ca). (B) Number of genes in *grvA* whose expression is altered relative to their expression in TW14359 plotted for each ontological group. The levels of expression of a total of 765 genes were altered by ≥2-fold; upregulation and downregulation are indicated.

O157:H7 strain TW14359 and its *grvA* isogenic derivative (TW14359 Δ*grvA*) were measured by RNA-seq analysis during exponential-phase growth (OD_{600} = 0.5) in LEE-inducing medium (DMEM).

In TW14359 Δ*grvA*, 765 genes were altered in expression >2-fold above the RPKM cutoff compared to their expression in TW14359; of these, 264 were upregulated and 501 were downregulated (Fig. 2; unpublished data). Known virulence-associated

genes made up 6% of the genes whose expression was altered (8 upregulated, 38 downregulated). The majority of these were LEE-encoded structural proteins and secreted effectors, consistent with the role of GrvA as a positive regulator of LEE expression (Fig. 2A and B). Twenty-one LEE genes were downregulated in TW14359 Δ*grvA*, including the LEE-encoded regulator *ler*, type III secretion translocon genes *espA*, *espB*, *espD*, and *eaeA* (encoding intimin), and the secreted effector *tir* (translocated intimin receptor) (Table 2). Five non-LEE-encoded effectors (*nleC*, *nleA*, *nleH2*, *nleF*, and *nleE*) were also downregulated in TW14359 Δ*grvA*. Genes encoded within the 15-kb genomic acid fitness island (AFI), the products of which are required for survival at low pH (48), were upregulated in TW14359 Δ*grvA* compared to their level of regulation in TW14359, in which the expression of AFI genes was barely detectable (Table 2). Most notably, genes of the glutamate-dependent acid resistance (GDAR) system were upregulated, including *gadA*, *gadE*, *gadW*, *gadX*, and the non-AFI-encoded *gadCB* operon. Thus, GrvA activates the LEE colonization mechanism while repressing acid resistance systems.

Twenty-six percent of the genes whose expression was altered (57 upregulated, 140 downregulated) had defined metabolic functions, suggesting a broader role for GrvA in regulation and fitness (Fig. 2B). This included genes for asparagine synthesis (*asnA*, *asnB*) and arginine degradation (*ast* operon) (Table 2). Nine percent (23 upregulated, 45 downregulated) were known or predicted regulators, and of these, global nitrogen metabolism regulators *ntrC* (also *glnG*) and *nac*, as well as *cbl* for sulfonate utilization, were downregulated. Eight percent (18 upregulated, 42 downregulated) were transport systems, and of these, glutamate (*glt* operon), glutamine (*gln* operon, and ammonium (*amtB*) transporters were downregulated. Thirty-seven percent (112 upregulated, 171 downregulated) had unknown or hypothetical functions (Table 2). Genes for zinc binding and transport, as well as resistance to zinc and other metals, were upregulated in TW14359 Δ*grvA*, including *znuA*, *znuCB*, *zinT* (formerly *yodA*), *rpmE2*, *rpmJ*, *ykgL*, and *yebA* (49–51).

Alterations in the expression of *astC* (arginine metabolism), *ler* (LEE regulator), *gadE* (acid resistance), and *znuC* (zinc transport) were validated by qRT-PCR. Complementation of *grvA* in *trans* (strain TW14359 Δ*grvA*/p*grvA*) largely restored wild-type expression for *ler* and *znuA* but only partially restored expression for *gadE* and *astC* (Table 3). Deletion of *rcsB* in TW14359 Δ*grvA* had no further significant impact on the expression of *ler* or any other targets tested (Table 3).

GrvA requires acid resistance regulator *gadE* for control of *ler* and adherence to intestinal cells. RNA-seq analysis revealed that deletion of *grvA* leads to the upregulation of *gadE*, a known transcriptional repressor of LEE-encoded regulator *ler* (52, 53). As such, the contribution of *gadE* to GrvA-dependent control of the LEE and adherence to cultured intestinal epithelial cells was examined.

During exponential-phase growth, *gadE* transcription is strongly repressed (54, 55). As such, the level of expression of *ler* in TW14359 Δ*gadE* only marginally increased compared to that in TW14359 (Fig. 3A). However, when *gadE* was constitutively expressed in both TW14359 Δ*grvA* and TW14359 Δ*grvA* Δ*gadE*, *ler* expression was reduced to the level of that observed in TW14359 Δ*grvA* (Fig. 3A) ($P < 0.05$).

The effect of the *grvA* and *gadE* interaction on *in vitro* adherence was determined using a competition assay. Strains were co-incubated in a 1:1 ratio with HT-29 intestinal cells, followed by plating and enumeration on MacConkey differential medium. For a control strain, *lacZ* was deleted from wild-type strain TW14359, and thus, the strain was Lac$^-$ and colonies on MacConkey agar were white, whereas the test strains (TW14359 and genetic derivatives) were Lac$^+$, producing pink colonies. For each test strain, an adherence index was determined from plate counts as the ratio of the number of CFU per milliliter for the test strain relative to the number of CFU per milliliter for the Lac$^-$ wild-type control strain.

In agreement with the *ler* expression data (Fig. 3A), the adherence index of TW14359 Δ*grvA* was significantly decreased compared to the adherence indexes of the wild type, TW14359 Δ*gadE*, TW14359 Δ*grvA* Δ*gadE*, and *grvA*-complemented strain TW14359 Δ*grvA*/p*grvA* ($P < 0.05$) but not that of type III secretion-defective strain TW14359 Δ*escN* (Fig. 3B). In strains where *gadE* was expressed constitutively, the adherence index was uniformly reduced to levels lower than those in all other strains, including TW14359 Δ*escN* ($P < 0.001$), suggesting that GadE may impact adherence in both a LEE-dependent and a LEE-independent manner (Fig. 3B). Only in strain TW14359 *grlR*::*kan*, in which the LEE is overexpressed (20), was the adherence index increased. The results of these experiments are consistent with the hypothesis that control of the LEE and adherence by GrvA are directed through the repression of *gadE*.

Negative regulation of *gadE* by GrvA requires GadW. GadE is a negative regulator of LEE expression, acting directly on the *LEE1* promoter to repress *ler* transcription (52, 53). In the preceding experiments, the upregulation of *ler* by GrvA was determined to correspond with *gadE* repression. Three discrete promoters have been shown to control transcription of the *gadE* gene in *E. coli* K-12 MG1655 (56). To better understand the molecular basis underlying the regulation of *gadE* promoters P1 to P3 by GrvA, the levels of transcription from four different *gadE lacZ* reporter constructs in TW14359 and TW14359 Δ*grvA* were compared during exponential-phase and stationary-phase growth in DMEM (Fig. 4A).

During exponential-phase growth, the level of transcription from *gadE-lacZ* containing all three *gadE* promoters (P1 through P3; Frag-1) was significantly increased in TW14359 Δ*grvA* compared to that in TW14359 ($P < 0.01$) (Fig. 4A) (55). Promoter activity from fragments containing the P1 or P3 promoter alone (Frag-2 and Frag-4, respectively) was also significantly higher in TW14359 Δ*grvA* ($P < 0.05$), while activity from the fragment containing only the P2 promoter (Frag-3) did not differ between TW14359 Δ*grvA* and TW14359. As anticipated, promoter activity from all fragments increased significantly during stationary-phase growth for TW14359 ($P < 0.01$) yet increased only slightly from P2 ($P < 0.05$). For TW14359 Δ*grvA*, promoter activity further increased only from P1 during stationary phase ($P < 0.01$) and was higher than the activity from P1 in TW14359 (Fig. 4A). This was predicted to be due to autoactivation of the P1 promoter by GadE (54), as deletion of *gadE* in TW14359 Δ*grvA* eliminated P1 activation (Frag-2) during exponential-phase growth but had no effect on P3 activity (Frag-4) (Fig. 4B). On the basis of these findings, it is suspected that the GrvA-dependent regulation of *gadE* transcription is directed solely through the P3 promoter.

Transcription from the *gadE* P3 promoter is directly controlled by the AFI-encoded regulators GadX and GadW (56), and RNA-seq analysis of TW14359 Δ*grvA* revealed the levels of expression of

TABLE 2 RNA-seq GrvA transcriptome

Gene identifier[a]	Gene name	Fold change in Δ*grvA* mutant[b]	RPKM[c]		Gene product description
			Wild type	Δ*grvA* mutant	
LEE pathogenicity island genes and genes for non-LEE-encoded effectors					
ECSP_4665	*espF*	−3.1	16.5	5.4	LEE-encoded effector
ECSP_4666	*orf29*	−3.4	11.9	3.5	LEE-encoded protein
ECSP_4667	*escF*	−5.8	15.5	2.7	LEE-encoded protein
ECSP_4668	*cesD2*	−8.3	46.3	5.6	Predicted chaperone
ECSP_4669	*espB*	−6.6	658.8	100.5	Secreted protein EspB
ECSP_4670	*espD*	−4.5	208.3	46.4	Secreted protein EspD
ECSP_4671	*espA*	−4.4	165.3	37.7	Secreted protein EspA
ECSP_4672	*sepL*	−4.5	46.8	10.3	LEE-encoded T3S component
ECSP_4673	*escD*	−4.0	4.8	1.2	LEE-encoded T3S component
ECSP_4674	*eaeA*	−4.8	75.0	15.8	Intimin adherence protein
ECSP_4675	*cesT*	−5.9	105.0	17.7	Chaperone
ECSP_4676	*tir*	−4.0	186.7	46.6	Translocated intimin receptor
ECSP_4677	*map*	−4.4	27.7	6.3	LEE-encoded effector
ECSP_4678	*cesF*	ND (−)[d]	3.4	$<10^{-4}$	Chaperone
ECSP_4679	*espH*	−4.8	22.9	4.8	LEE-encoded effector
ECSP_4680	*escQ*	−1.9	9.7	5.0	LEE-encoded protein
ECSP_4681	*orf16*	−8.1	14.7	1.8	LEE-encoded protein
ECSP_4682	*orf15*	−1.2	5.9	4.9	LEE-encoded protein
ECSP_4683	*escN*	−2.7	16.8	6.2	LEE-encoded ATPase
ECSP_4684	*escV*	−2.3	11.6	5.1	LEE-encoded protein
ECSP_4685	*orf12*	−1.3	2.6	2.0	LEE-encoded protein
ECSP_4686	*espZ*	−3.5	78.4	22.6	LEE-encoded effector
ECSP_4687	*escI*	−1.3	12.7	9.9	LEE-encoded protein
ECSP_4688	*escJ*	−2.8	18.4	6.6	LEE-encoded protein
ECSP_4689	*sepD*	−1.3	2.2	1.7	LEE-encoded protein
ECSP_4690	*escC*	−1.7	7.8	4.5	T3S needle complex subunit
ECSP_4691	*cesD*	−1.1	6.5	6.0	LEE-encoded protein
ECSP_4692	*grlA*	−1.1	15.3	13.8	Global regulator of LEE, activator
ECSP_4693	*grlR*	−1.9	11.4	5.9	Global regulator of LEE, repressor
ECSP_4694	*etgA*	−1.5	1.3	0.9	Lytic transglycosylase
ECSP_4695	*escU*	ND (−)	1.6	$<10^{-4}$	LEE-encoded protein
ECSP_4696	*escT*	ND (−)	2.0	$<10^{-4}$	LEE-encoded protein
ECSP_4697	*escS*	4.0	0.6	2.4	LEE-encoded protein
ECSP_4698	*escR*	−1.6	4.3	2.6	LEE-encoded protein
ECSP_4699	*escL*	−2.9	12.2	4.2	LEE-encoded protein
ECSP_4700	*orf4*	−1.8	10.3	5.6	LEE-encoded protein
ECSP_4701	*cesA*	−1.0	2.2	2.2	Chaperone
ECSP_4702	*escE*	−1.1	18.3	16.2	LEE-encoded protein
ECSP_4703	*ler*	−2.5	55.7	22.7	LEE encoded regulator
ECSP_4704	*espG*	−3.7	4.5	1.2	LEE encoded effector
ECSP_4705	*rorf1*	−1.0	0.6	0.6	LEE-encoded protein
ECSP_0061	*espY1*	2.0	0.3	0.6	Non-LEE-encoded effector
ECSP_0866	*nleC*	−2.3	4.3	1.9	Non-LEE-encoded effector
ECSP_0868	*nleD*	−1.7	7.3	4.4	Non-LEE-encoded effector
ECSP_1702	*nleA*	−4.0	1.5	0.4	Non-LEE-encoded effector
ECSP_1704	*nleH2*	2.2	3.8	8.6	Non-LEE-encoded effector
ECSP_1705	*nleF*	−3.7	16.2	4.4	Non-LEE-encoded effector
ECSP_3954	*nleE*	−4.0	2.6	0.6	Non-LEE-encoded effector
Metabolism and acid resistance genes					
ECSP_0704	*gltL*	−3.9	149.2	38.4	Glutamate and aspartate transporter
ECSP_0705	*gltK*	−3.0	31.2	10.3	Glutamate and aspartate transporter
ECSP_0706	*gltJ*	−2.5	29.9	12.0	Glutamate and aspartate transporter
ECSP_0707	*gltI*	−3.0	156.1	52.4	Glutamate and aspartate transporter
ECSP_0906	*glnQ*	−2.3	277.1	118.0	Glutamine transporter subunit
ECSP_0907	*glnP*	−2.3	32.1	13.8	Glutamine transporter subunit
ECSP_0908	*glnH*	−1.5	141.1	93.5	Glutamine transporter subunit
ECSP_1977	*gadC*	15.6	1.3	20.5	Glutamate:gamma-aminobutyric acid antiporter

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TABLE 2 (Continued)

Gene identifier[a]	Gene name	Fold change in Δ*grvA* mutant[b]	RPKM[c]		Gene product description
			Wild type	Δ*grvA* mutant	
ECSP_1978	*gadB*	14.6	4.1	59.5	Glutamate decarboxylase B
ECSP_2312	*astE*	−4.0	9.7	2.4	Succinylglutamate desuccinylase
ECSP_2313	*astB*	−5.9	10.9	1.8	Succinylarginine dihydrolase
ECSP_2314	*astD*	−9.1	18.3	2.0	Succinylglutamic semialdehyde dehydrogenase
ECSP_2315	*astA*	ND (−)	25.5	$<10^{-4}$	Arginine succinyltransferase
ECSP_2316	*astC*	−66.0	52.4	0.8	Succinylornithine transaminase
ECSP_2329	*gdhA*	−1.8	53.4	23.0	Glutamate dehydrogenase
ECSP_3185	*argT*	−3.5	89.1	25.3	Lysine/arginine/ornithine transporter subunit
ECSP_4065	*uxaA*	1.7	12.6	21.4	Altronate hydrolase
ECSP_4066	*uxaC*	1.8	7.8	13.6	Uronate isomerase
ECSP_4486	*slp*	2.0	37.4	74.3	Outer membrane lipoprotein
ECSP_4498	*hdeB*	ND (+)	$<10^{-4}$	13.6	Acid resistance protein
ECSP_4499	*hdeA*	ND (+)	$<10^{-4}$	134.4	Acid resistance protein
ECSP_4500	*hdeD*	ND (+)	$<10^{-4}$	11.7	Acid resistance protein
ECSP_4501	*gadE*	ND (+)	$<10^{-4}$	10.8	GDAR activator
ECSP_4502	*mdtE*	ND (+)	$<10^{-4}$	11.6	Multidrug resistance efflux transporter
ECSP_4503	*mdtF*	ND (+)	$<10^{-4}$	12.9	Multidrug resistance efflux transporter
ECSP_4504	*gadW*	ND (+)	$<10^{-4}$	9.1	DNA-binding transcriptional activator
ECSP_4505		1.0	$<10^{-4}$	0.0	Hypothetical protein
ECSP_4506	*gadX*	ND (+)	$<10^{-4}$	5.0	DNA-binding transcriptional dual regulator
ECSP_4507	*gadA*	ND (+)	$<10^{-4}$	18.3	Glutamate decarboxylase A
ECSP_4508	*yhjA*	1.0	$<10^{-4}$	$<10^{-4}$	Cytochrome *c* peroxidase
ECSP_4509	*treF*	ND (+)	$<10^{-4}$	3.4	Cytoplasmic trehalase
ECSP_4510	*yhjB*	1.0	$<10^{-4}$	$<10^{-4}$	DNA-binding response regulator
ECSP_4511	*yhjC*	ND (+)	$<10^{-4}$	2.6	DNA-binding transcriptional regulator
ECSP_4512	*yhjD*	ND (+)	$<10^{-4}$	5.1	Conserved inner membrane protein
ECSP_4513	*yhjE*	87.3	0.2	16.3	Predicted transporter
ECSP_4921		ND	2.7	$<10^{-4}$	Hypothetical protein
ECSP_4922		−1.1	78.6	74.4	Hypothetical protein
ECSP_4923	*glnG*	−3.7	83.9	22.4	DNA-binding response regulator
ECSP_4924	*glnL*	−3.7	117.1	31.6	Sensory histidine kinase
ECSP_4925	*glnA*	−4.3	745.3	172.4	Glutamine synthetase
ECSP_0518	*glnK*	ND (+)	143.4	$<10^{-4}$	Nitrogen assimilation regulatory protein
ECSP_0519	*amtB*	−74.8	185.6	2.7	Ammonium transporter
ECSP_4793	*asnC*	−2.0	5.2	2.6	DNA-binding transcriptional regulator
ECSP_4794	*asnA*	−5.0	2,647.6	532.5	Asparagine synthetase A
ECSP_0721	*asnB*	−7.6	688.2	90.7	Asparagine synthetase B
ECSP_2651	*cbl*	−4.8	14.1	2.9	DNA-binding transcriptional activator
ECSP_2652	*nac*	−63.5	32.0	0.5	DNA-binding transcriptional dual regulator
ECSP_3790	*galR*	−2.1	13.2	6.4	DNA-binding transcriptional repressor
ECSP_3791	*lysA*	−4.3	23.1	5.4	Diaminopimelate decarboxylase
ECSP_3792	*lysR*	−2.8	2.8	1.0	DNA-binding transcriptional dual regulator
Metal stress response and import/export genes					
ECSP_0334	*ykgL*	ND (+)	$<10^{-4}$	1.4	Predicted protein
ECSP_0335	*rpmJ*	170.7	1.7	284.6	50S ribosomal protein L36
ECSP_0336	*rpmE2*	565.7	0.6	346.5	Zn(II)-responsive ribosomal protein
ECSP_0622	*cusS*	−1.3	7.6	5.8	Sensory histidine kinase
ECSP_0623	*cusR*	1.1	36.3	38.9	DNA-binding response regulator
ECSP_0624	*cusC*	−1.0	15.6	15.0	Copper/silver efflux system
ECSP_0625	*cusF*	−1.8	47.5	26.5	Periplasmic copper-binding protein
ECSP_0626	*cusB*	−1.3	111.6	82.8	Copper/silver efflux system
ECSP_0627	*cusA*	−1.4	67.5	48.0	Copper/silver efflux system
ECSP_0731	*fur*	1.7	38.2	64.5	DNA-binding regulator
ECSP_2430	*yebA*	4.5	17.0	75.9	Predicted peptidase
ECSP_2431	*znuA*	17.7	13.8	244.0	High-affinity zinc uptake system
ECSP_2432	*znuC*	2.5	4.5	18.9	High-affinity zinc uptake system
ECSP_2433	*znuB*	4.2	4.6	11.4	High-affinity zinc uptake system

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TABLE 2 (Continued)

Gene identifier[a]	Gene name	Fold change in Δ*grvA* mutant[b]	RPKM[c]		Gene product description
			Wild type	Δ*grvA* mutant	
ECSP_2579	*zinT*	163.7	2.0	328.1	Conserved metal-binding protein
ECSP_4263	*zntR*	−1.1	11.4	9.9	DNA-binding transcriptional activator
ECSP_4425	*zntA*	−1.4	19.9	14.3	Zinc, cobalt, and lead efflux system
Genes for other functions					
ECSP_1968	*ddpF*	1.1	2.3	2.5	D-Ala–D-Ala transporter subunit
ECSP_1969	*ddpD*	1.5	1.0	1.4	D-Ala–D-Ala transporter subunit
ECSP_1970	*ddpC*	1.6	1.3	2.1	D-Ala–D-Ala transporter subunit
ECSP_1971	*ddpB*	−6.5	3.1	0.5	D-Ala–D-Ala transporter subunit
ECSP_1972	*ddpA*	−28.7	9.3	0.3	D-Ala–D-Ala transporter subunit
ECSP_1973	*ddpX*	ND (−)	7.3	$<10^{-4}$	D-Ala–D-Ala dipeptidase
ECSP_2574	*yedV*	−1.8	2.7	1.4	Sensory kinase
ECSP_2575	*yedW*	1.0	48.2	26.5	DNA-binding regulator
ECSP_5434	*yjiA*	2.8	12.3	34.1	Predicted GTPase
ECSP_5435	*yjiX*	27.8	0.7	19.5	Conserved protein
ECSP_5436	*yjiY*	3.8	64.6	246.2	Predicted protein
ECSP_2492		−2.4	1,462.1	620.0	Hypothetical protein
ECSP_4094		−1.9	1,576.0	841.5	Hypothetical protein

[a] Based on EHEC O157:H7 strain TW14359 (GenBank accession number NC_013008.1).
[b] Fold change was calculated as the wild-type RPKM value divided by the mutant strain RPKM value.
[c] RPKM, reads per kilobase per million mapped reads.
[d] ND, not determined; (+), upregulated; (−), downregulated.

both genes to be elevated compared to their levels of expression in TW14359 (Table 2). It was thus predicted that repression of P3 by GrvA was mediated through one or both of these regulators. To test this, the effect of *gadX* and *gadW* deletion in TW14359 Δ*grvA* on P3 activity during exponential-phase growth (OD_{600} = 0.5) in DMEM was measured. Only the deletion of *gadW* was observed to reduce the level of transcription from P3 (Fig. 4C). Activity from P3 in TW14359 Δ*gadW* and TW14359 Δ*grvA* Δ*gadW* was reduced significantly compared to that in TW14359 Δ*grvA* ($P = 0.01$ and 0.03, respectively) but not compared to that in TW14359. Conversely, activity from P3 was slightly but significantly increased in TW14359 Δ*grvA* Δ*gadX* compared to that in TW14359 Δ*grvA* ($P = 0.01$) (Fig. 4C). Taken together, these data indicate that GrvA indirectly represses transcription from the *gadE* P3 promoter during exponential-phase growth in a manner that is dependent on *gadW*.

GrvA is a novel repressor of glutamate-dependent acid resistance. The transcription of GDAR genes is growth phase dependent; expression is tightly controlled and low during exponential-phase growth but increases markedly as cells transition into stationary phase (57). Correspondingly, exponential-phase cultures are generally acid susceptible, while stationary-phase cultures are acid resistant. Since deletion of *grvA* was shown to upregulate GDAR genes (*gadA*, *gadBC*, *gadE*, *gadW*, *gadX*) during exponential-phase growth, the contribution of GrvA to GDAR was determined for exponential-phase cultures and was compared to that of the GDAR phenotype of stationary-phase cultures. In addition, since RcsB activates *grvA* transcription and is a dual regulator of GDAR system genes in *E. coli* K-12 (28, 58, 59), the interactions of *rcsB* and *grvA* in expression of the GDAR phenotype in EHEC O157:H7 were examined.

As expected, no colonies of wild-type TW14359 grown to exponential phase could be recovered on LBA following a 1-h challenge in acidified (pH 2) EG medium (Fig. 5, top). However, for strain TW14359 Δ*grvA*, in which GDAR genes are upregulated during exponential-phase growth, 400 CFU/ml, corresponding to 9% of the original inoculum, was recovered. Complementation of TW14359 Δ*grvA* with *grvA* restored acid sensitivity to wild-type levels. GDAR was abrogated following deletion of *rcsB* in TW14359 Δ*grvA*, and complementation with *rcsB* restored GDAR in TW14359 Δ*grvA* Δ*rcsB* but not in TW14359 Δ*rcsB*.

In stationary phase, during which GDAR is actively expressed (57), deletion and complementation of *grvA* had no effect on survival in acid, with 100% of the initial inoculum being recovered following a 1-h challenge in acidified EG medium (Fig. 5, bottom).

TABLE 3 qRT-PCR validation of RNA-seq data

Strain	Expression units[a]			
	ler	*gadE*	*astC*	*znuA*
TW14359	1,054.77 (51.81)	0.18 (0.06)	111.56 (1.55)	8.02 (2.34)
Δ*grvA* mutant	78.28 (21.68)	61.11 (8.22)	1.21 (0.67)	551.15 (150.90)
Δ*rcsB* Δ*grvA* mutant	127.97 (33.65)	87.87 (4.10)	2.5 (1.49)	622.03 (46.05)
Δ*grvA* p*grvA* mutant	556.98 (180.36)	12.94 (0.81)	6.87 (2.24)	20.46 (8.28)

[a] For each gene, expression units relative to the level of *rrsH* expression were calculated, and standard deviations are in parentheses. Gene name identifiers are based on EHEC strain TW14359 (GenBank accession number NC_013008.1).

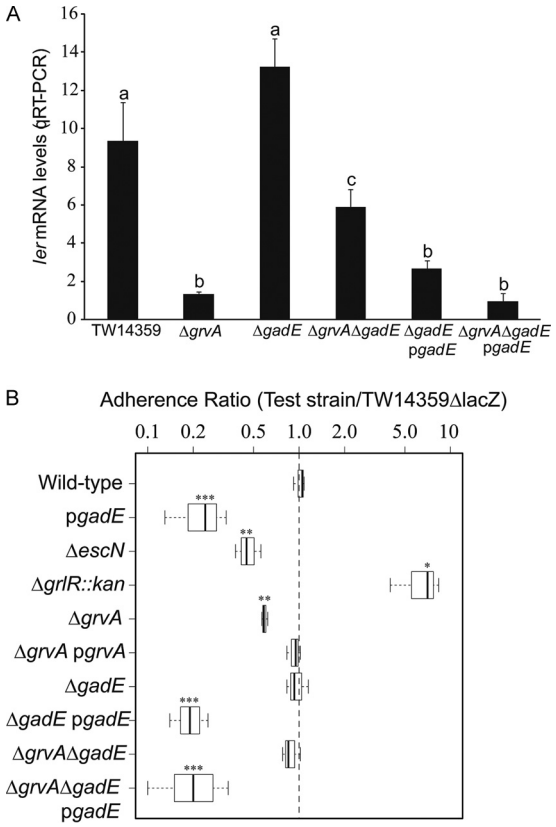

FIG 3 GrvA requires *gadE* for control of LEE-dependent adherence. (A) *ler* transcript levels determined by qRT-PCR plotted for TW14359, TW14359 Δ*grvA*, TW14359 Δ*gadE*, TW14359 Δ*grvA* Δ*gadE*, and complemented strains TW14359 Δ*gadE*/p*gadE* and TW14359 Δ*grvA* Δ*gadE*/p*gadE* grown in DMEM (OD_{600} = 0.5). Plots which differ by lowercase letter differ significantly by Tukey's HSD test following a significant *F* test ($n \geq 3$, $P < 0.05$). Error bars denote standard deviations. (B) Adherence ratio box plot for test strains (TW14359 and genetic derivatives) relative to control strain TW14359 Δ*lacZ* when cultured with HT-29 colonic epithelial cells (see the text for details). Box plot boundaries represent the 25th and 75th percentiles, whiskers represent the maximum and minimum values, and the mean adherence ratio is given by the horizontal line. Asterisks denote a significant difference compared with the results for TW14359, as determined by *t* test ($n \geq 3$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Thus, control of GDAR by GrvA is relegated to exponential-phase growth. In keeping with the requirement for *rcsB* in stationary-phase GDAR, no growth was observed for TW14359 Δ*rcsB* and TW14359 Δ*grvA* Δ*rcsB* unless they were complemented with *rcsB* (Fig. 5, bottom).

DISCUSSION

This study has examined aspects of *grvA* transcriptional regulation, has defined the GrvA regulon, and has identified genetic determinants underlying GrvA-dependent control of discrete pathogenic mechanisms in EHEC. RcsB, the response regulator of Rcs phosphorelay in *E. coli*, is predicted to directly activate transcription of *grvA*, leading to the upregulation of LEE-dependent adherence and repression of glutamate-dependent acid resistance (Fig. 6). While it is not yet clear what *cis*-acting element(s) is required for RcsB-dependent activation of *grvA*, this study demonstrated the binding of RcsB to a *grvA* promoter fragment containing two putative tandem RcsB binding sites located proximal to a predicted σ^{70} −35 site. These sites share some homology with the RcsAB box consensus sequence described for *rcsA*, *wza*, and *flhDC* promoters (30, 60), yet RcsA, which is highly unstable at elevated temperatures (27), is not required for RcsB-dependent regulation of the LEE (21), and RcsAB generally bind distal to and upstream of the promoter core (31, 61, 62). The binding of RcsB to *grvA* promoter fragments is more consistent with regulation as a homodimer. RcsB homodimers bind a consensus sequence similar to the RcsAB box and proximal to the −35 site. Of the two putative RcsB binding sites in the *grvA* promoter, the upstream site (Fig. 1B, Box 1) shares similarities with homodimer sites described for *osmC* and *rprA* promoters (63). RcsB has also been shown to negatively regulate *grvA* expression. In strains of *E. coli* O157:H7 harboring a 49-bp deletion in *rcsB*, *trans*-complementation with wild-type *rcsB* decreased *grvA* expression (64). This was observed, however, only during culture at 28°C and correlates with reduced *grvA* expression during growth at 14°C and 25°C (65). Together, this suggests that regulation of *grvA* by RcsB is temperature sensitive. There is precedent for this, as repression of *flhDC* transcription by RcsB occurs at both low and high temperatures. At low temperatures, RcsB partners with RcsA (30), whereas at high temperatures, the LEE-encoded regulator GrlA is required (20).

Overexpression of *grvA* increases transcription from the *LEE1* promoter and correspondingly leads to increased adherence (21). Consistent with this, RNA-seq analysis of TW14359 Δ*grvA* in this study revealed the reduced expression of genes belonging to both the *LEE1* and the *LEE2-LEE3* operons, including LEE-encoded regulator *ler*, T3S structural genes (*esp* genes), and the translocated intimin receptor (*tir*) gene. This study is the first to show that *grvA* deletion downregulates non-LEE-encoded T3S effector genes (*nle* genes). These proteins broadly impact host signaling cascades through inhibition of NF-κB (NleC, NleE, NleD, and NleH2) (66–68) and caspase activation (NleF) (69), as well as interfere with host vesicle trafficking (NleA) (70). How these *nle* genes are regulated by GrvA is unknown. One hypothesis is through its regulatory effects on LEE expression. Both LEE-encoded activators Ler and GrlA regulate *nleA* expression (71–73). Moreover, LEE-encoded GrlR has been shown to repress *nleB* and *nleH* (71). However, non-LEE-encoded regulators involved in quorum sensing (QseA) and nucleoid structuring (H-NS) have also been implicated in the control of *nle* gene expression (71, 74). Whatever the mechanism, these findings suggest a more comprehensive role for GrvA in EHEC pathogenesis that includes the coordination of T3S-dependent colonization with immune subversion.

This study identifies GrvA to be a new regulator of glutamate-dependent acid resistance (GDAR) genes (*gad* genes) (Fig. 6). Deletion of *grvA* led to the upregulation of *gad* regulatory (*gadE*, *gadW* and *gadX*) and enzymatic/structural (*gadA* and *gadBC*) genes and corresponded with a 100-fold increase in acid survival by the GDAR mechanism. These findings greatly expand the role for GrvA in the pathogenic behavior of EHEC to include the control genes essential for the transmission of this pathogen in acidic food matrices, gastric passage, and a low oral infectious dose. Since *grvA* is needed for full bicarbonate induction of the LEE (20), it is plausible that it helps to coordinate expression of the LEE and GDAR systems upon entry into the intestine (Fig. 6).

Genes with no direct role in virulence were also shown to be regulated by GrvA. Most notably, *glnG* (also *ntrC*) and *nac* expression was positively regulated by GrvA. Nitrogen regulatory pro-

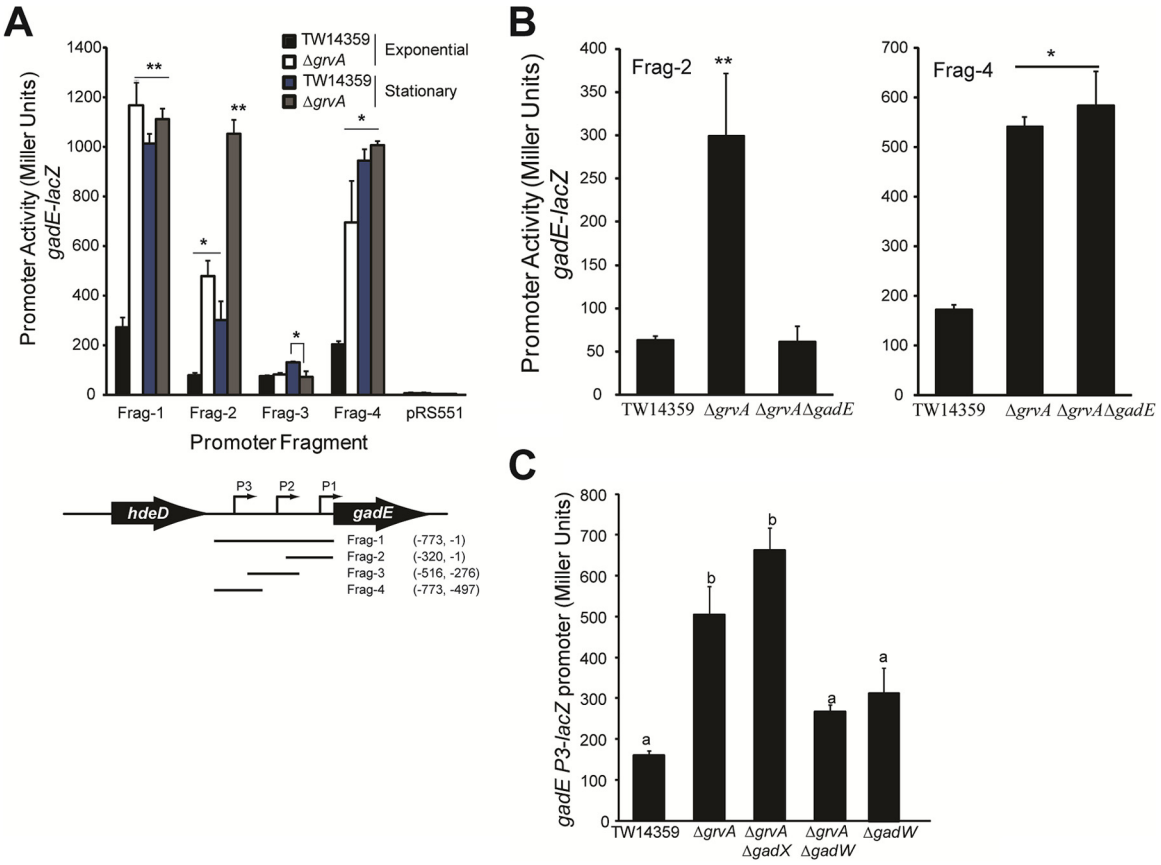

FIG 4 GrvA represses *gadE* transcription through GadW, determined from β-galactosidase activity (in Miller units) for *gadE-lacZ* promoter fusions in TW14359 and genetic derivatives. (A) (Top) Activity (in Miller units) for *gadE-lacZ* promoter fragments Frag-1 through Frag-4 and the vector control (pRS551) plotted for TW14359 and TW14359 Δ*grvA* during exponential-phase (OD_{600} = 0.5) and stationary-phase (OD_{600} = 2) growth in DMEM. (Bottom) Cartoon of the *gadE* promoter and flanking regions. Promoter (P1 to P3) positions and the positions of each cloned promoter fragment (Frag-1 through Frag-4) relative to position +1 of the initiation codon for the *gadE* ORF are indicated. (B) Activity (in Miller units) plotted for *gadE-lacZ* promoter fragments Frag-2 (left) and Frag-4 (right) in TW14359 and mutant derivatives. For panels A and B, asterisks denote significant differences by *t* test ($n \geq 3$; *, $P < 0.05$; **, $P < 0.01$). Bars denote standard deviations. (C) Activity (in Miller units) plotted for the *gadE-lacZ* P3 promoter in TW14359 and mutant derivatives. Plots which differ by lowercase letter differ significantly by Tukey's HSD test following a significant *F* test ($n \geq 3$, $P < 0.05$).

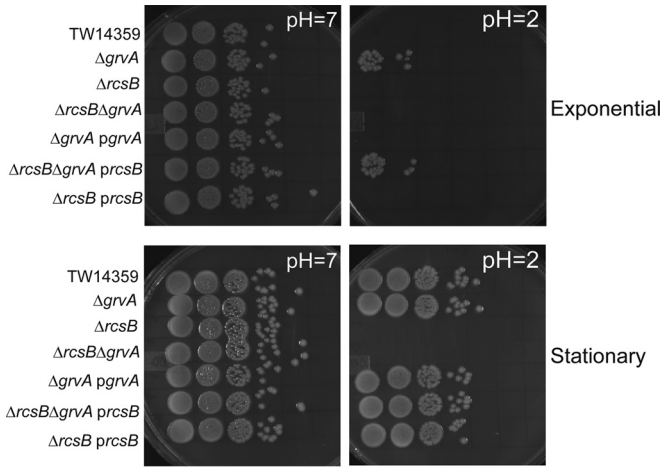

FIG 5 GrvA is a growth phase-dependent repressor of acid resistance. Representative colony-forming units on LB agar for TW14359 and derivative strains following a 1-h challenge in EG medium (pH 7 versus pH 2) are shown. Cultures were tested for acid resistance during exponential-phase (top) and stationary-phase (bottom) growth in DMEM. See the text for details.

tein C (NtrC) is a transcriptional activator of nitrogen assimilation control (Nac), and together these regulators are responsible for coordinating the expression of genes for nitrogen metabolism under limiting conditions (75). That *ntrC* and *nac* are positively controlled by GrvA is consistent with the reduced expression of operons for the utilization of glutamate (*glt*), glutamine (*gln*), arginine (*ast*), and asparagine (*asn*), as well as for the transport of ammonium (*amtB*) observed in the TW14359 Δ*grvA* background. How nitrogen availability influences GrvA-dependent control of these mechanisms and the importance of *grvA* to nitrogen assimilation are unknown.

GrvA was shown to activate LEE-encoded master regulator *ler* through downregulation of *gadE*, the product of which directly represses *ler* transcription in EHEC (52, 53, 76). Negative control of *gadE* by GrvA is predicted to occur indirectly through the P3 promoter, requiring an intact *gadW* (Fig. 6). This is supported by both *gadE-lacZ* promoter fusions and RNA-seq analysis in this study. GadW is an AraC-family regulator that acts as a homodimer or, when partnered with GadX, to control the acid fitness genes *gadBC*, *hdeA*, *hdeB*, and *gadE* (54, 55, 77, 78). The details of how GrvA regulates transcription are not yet known. For

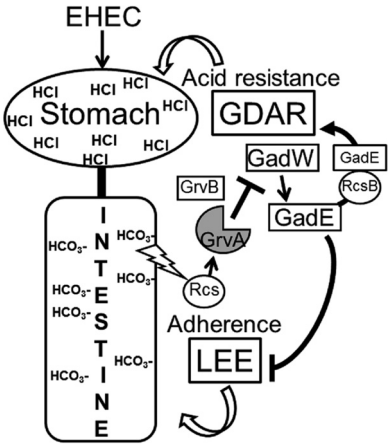

FIG 6 Model predicting GrvA-dependent regulation of acid resistance and LEE-dependent adherence. During exponential-phase growth, GrvA represses acid resistance (GDAR) and activates LEE-dependent adherence. This requires the negative regulation of *gadW* by GrvA. As GadW is a direct activator of *gadE*, the central regulator of GDAR, its repression by GrvA downregulates GDAR gene expression (*gadE*, *gadA*, *gadBC*) and acid resistance. GadE also directly represses the LEE-encoded regulator *ler*, the product of which activates the LEE (5 operons encoding the T3S, other regulators, and toxic effectors). The repression of GadW/GadE by GrvA leads to activation of the LEE and LEE-dependent adherence. Bicarbonate in the intestine is hypothesized to stimulate GrvA-dependent control of GDAR and the LEE in a manner requiring the Rcs phosphorelay system. RcsB activates GDAR as a heterodimer with GadE. The role for GrvB in this regulatory pathway is unknown. GrvA regulates the expression of >700 genes in EHEC O157:H7 strain TW14359. The model is inferred from the results of experiments performed in this and previous studies (20, 21, 52, 53, 55). See the text for further details. GDAR, glutamate-dependent acid resistance; LEE, locus of enterocyte effacement.

the regulation of *gadW*, there are two predicted promoters encoded in tandem and just upstream of the *gadW* ($gadW_{P1}$ and $gadW_{P2}$) (77). It is suspected that $gadW_{P1}$ is the target of GrvA repression, as transcripts from this promoter, but not those from $gadW_{P2}$, were increased in TW14359 Δ*grvA* by RNA-seq (data not shown). Multiple *trans*-acting factors are known to directly influence *gadW* transcription, including GadE, PhoP, and SdiA (activators) (79–81), as well as GadX, GadW, RutR, Fnr, and H-NS (repressors) (77, 82–84). It is unlikely that *gadX* contributes to GrvA-dependent repression of *gadW*, as *gadX* mutation in TW14359 Δ*grvA* was not observed to further alter *gadE* expression. Save for *gadE*, no other known regulators of *gadW* were modified in expression by RNA-seq analysis, and a binding consensus sequence for GrvA has yet to be defined. GrvA shares some homology (29% identity, 79/270 amino acids) with MarT of *Salmonella enterica* serotype Typhimurium (85–87), most notably, the first 150 residues of the N terminus containing a winged-helix DNA-binding domain. *marT* is encoded on *Salmonella* pathogenicity island 3 (SPI3), and its product is a direct transcriptional activator of the *misL* autotransporter, which binds fibronectin and increases intestinal colonization and invasiveness (88, 89). On the basis of its similarity to CadC of *E. coli*, MarT is predicted to activate *misL* by relieving H-NS-mediated repression (33). If the regulation of *gadW* by GrvA is by a direct mechanism, however, it is unlikely that H-NS is involved, as deletion of *grvA* increases *gadW* expression. Future studies will be aimed at identifying *cis* elements for GrvA-directed transcriptional regulation.

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