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The Type Three Secretion System 2-Encoded Regulator EtrB Modulates Enterohemorrhagic *Escherichia coli* Virulence Gene Expression

Deborah H. Luzader,[a] Graham G. Willsey,[b] Matthew J. Wargo,[b] Melissa M. Kendall[a]

Department of Microbiology, Immunology, and Cancer Biology, University of Virginia School of Medicine, Charlottesville, Virginia, USA[a]; Department of Microbiology and Molecular Genetics, University of Vermont College of Medicine, Burlington, Vermont, USA[b]

Enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) is a foodborne pathogen that causes bloody diarrhea and hemolytic uremic syndrome throughout the world. A defining feature of EHEC pathogenesis is the formation of attaching and effacing (AE) lesions on colonic epithelial cells. Most of the genes that code for AE lesion formation, including a type three secretion system (T3SS) and effectors, are carried within a chromosomal pathogenicity island called the locus of enterocyte effacement (LEE). In this study, we report that a putative regulator, which is encoded in the cryptic *E. coli* type three secretion system 2 (ETT2) locus and herein renamed EtrB, plays an important role in EHEC pathogenesis. The *etrB* gene is expressed as a monocistronic transcript, and EtrB autoregulates expression. We provide evidence that EtrB directly interacts with the *ler* regulatory region to activate LEE expression and promote AE lesion formation. Additionally, we mapped the EtrB regulatory circuit in EHEC to determine a global role for EtrB. EtrB is regulated by the transcription factor QseA, suggesting that these proteins comprise a regulatory circuit important for EHEC colonization of the gastrointestinal tract.

Enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) is a foodborne pathogen that causes severe bloody diarrhea, which may be associated with complications, including hemolytic uremic syndrome (1). A defining feature of EHEC infection is the formation of attaching and effacing (AE) lesions on colonic enterocytes. AE lesions are characterized by intimate attachment of EHEC to epithelial cells, rearrangement of the actin cytoskeleton, and effacement of the microvilli (2–4). Most of the genes that mediate AE lesion formation are carried within the locus of enterocyte effacement (LEE) pathogenicity island (5). The LEE is comprised of five major operons, *LEE1* to *LEE5* (6–8), which encode a type three secretion system (T3SS) (*E. coli* type three secretion system-1 [ETT1]) (2), an adhesin (intimin) (3) and its receptor (Tir) (4), and secreted effectors (9–13). The LEE also encodes regulatory proteins, including the *LEE1*-encoded Ler that activates expression of all of the LEE genes (8) as well as GrlA and GrlR, which positively and negatively influence LEE expression, respectively (14–16). Additionally, the LEE-encoded T3SS translocates effector proteins encoded outside the LEE that are also important for virulence (15, 17–23).

In addition to the LEE-encoded T3SS, EHEC carries another locus that encodes a nonfunctional T3SS, named *E. coli* T3SS-2 (ETT2) (24, 25), which shares homology to the *Salmonella* T3SS-1 (26). The ETT2 pathogenicity island encodes five predicted or characterized transcription factors. A study by Zhang et al. showed that two of these, EtrA and EivF, repress LEE expression and adherence to epithelial cells, whereas YgeH displayed no regulatory phenotype (27). The other ETT2-encoded putative transcription factors, YqeI and YgeK, herein renamed EtrB, have not been characterized.

EHEC controls expression of virulence traits through complex regulatory circuits that are responsive to metabolites, host hormones, and bacterial signaling molecules, in addition to other environmental cues (28–37). For example, expression of the transcription factor QseA is induced through bacterial cell signaling as well as by ethanolamine, an abundant metabolite in the intestine (33, 38). QseA is an LysR-type regulator that activates LEE expression by directly binding the *ler* promoter as well as promoting *grlA* transcription (16, 38–40). Additionally, QseA controls expression of genes located in several O-islands (which are regions of the genome not carried in *E. coli* K-12 strains [24, 25]), and importantly, QseA positively regulates *etrB* expression (39). Because ETT2-encoded proteins have been shown to influence virulence and because genetic data indicate that *etrB* is part of the QseA regulatory cascade, we hypothesized that EtrB plays a role in modulating EHEC virulence gene expression. Our findings indicate that EtrB activates LEE expression, not only through direct regulation but also by repressing expression of *eivF* and *etrA*. Moreover, we mapped the EtrB regulon and report that EtrB also modulates expression of genes encoding distinct functions, including the non-LEE-encoded effector NleA, a fimbrial adhesin, a small RNA (sRNA), and maltose and tryptophan metabolism.

MATERIALS AND METHODS

Strains, plasmids, growth conditions, and recombinant DNA techniques. Strains and plasmids used in this study are listed in Table 1. Standard methods were used to perform plasmid purification, PCR, ligation,

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Address correspondence to Melissa M. Kendall, melissakendall@virginia.edu.

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
Strains		
86-24	Wild-type EHEC (serotype O157:H7)	51
DL01	86-24 *etrB* mutant	This study
DL02	*etrB* mutant containing pGEN-MCS	This study
DL03	DL01 containing plasmid pDL01 (*etrB* complement)	This study
DL04	86-24 containing pGEN-MSC	This study
DL05	86-24 containing pDL03 (*etrB*::*lux* fusion)	This study
DL06	*etrB* mutant containing pDL03 (*etrB*::*lux* fusion)	This study
VS145	86-24 *qseA* mutant	38
VS151	VS145 with plasmid pVS150 (*qseA* complement)	38
BL21(DE3)	F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)	Invitrogen
Plasmids		
pKD3	pANTSλ derivative containing FRT-flanked kanamycin resistance	41
pKD46	Lambda red recombinase expression plasmid	41
pCP20	Temperature-sensitive replication and thermal induction of FLP synthesis	41
pGEN-MCS	Cloning vector	42
pGEN-luxCDABE	P_{em7}-*lux*; synthetic promoter, constitutive *lux* expression (Amp^r)	42
pMAL-c5x	Cloning vector	NEB
pVS150	EHEC 86-24 *qseA* in pACYC177	38
pMK08	EHEC 86-24 *qseA* in pET28	39
pDL01	EHEC 86-24 *etrB* in pGEN	This study
pDL02	EHEC 86-24 *etrB* in pMAL-C5x	This study
pDL03	EHEC 86-24 *etrB* in pGEN-*luxCDABE*	This study

restriction digests, transformations, and gel electrophoresis. Luria-Bertani (LB) broth (Invitrogen) or Dulbecco's modified Eagle's medium (DMEM; Invitrogen) was used to grow bacteria. For experiments with the *qseA* deletion strain (VS145), bacteria were grown overnight in LB broth, diluted 1:100 in low-glucose DMEM, and then grown at 37°C, aerobically to late exponential growth phase (optical density at 600 nm [OD_{600}] of 1.0). For all other experiments, bacteria were grown overnight in LB broth, diluted 1:100 in low-glucose DMEM, and grown for 6 h statically at 37°C under a 5% CO_2 atmosphere. Streptomycin was added to overnight cultures of EHEC at a final concentration of 50 μg/ml. Overnight cultures of strains carrying pGEN, pDL01, or pDL03 contained ampicillin to a final concentration of 100 μg/ml. The nonpolar EHEC 86-24 *etrB* mutant (DL01) was constructed using lambda red mutagenesis (41). The mutant was complemented with *etrB* under the endogenous promoter cloned into pGEN-MCS (42) (Addgene MTA) using the restriction enzymes HindIII and NcoI (NEB). When complement data are shown, the wild-type (WT) and Δ*etrB* strains contain empty vector controls. Strains and plasmids were confirmed by DNA sequencing. Primers used to generate the *etrB* deletion and complemented strains are listed in Table 2.

Measurement of *etrB* expression. The plasmid pGEN-luxCDABE (42) was used to create the *etrB* expression plasmid named pDL03. For this, approximately 332 bp of the *etrB* promoter region was inserted upstream of *luxCDABE* using the restriction enzymes PmeI and SnaBI (NEB). Luminescence was measured using a Victor Wallac luminometer (Perkin-Elmer). Luminescence was corrected for the OD_{600} value for each condition. Statistical significance was determined by Student's *t* test.

RNA extraction and qRT-PCR. RNA purification and quantification of RNA transcription were performed as described previously (43). RNA was extracted from three biological replicate cultures of each strain/condition using a RiboPure Bacteria RNA isolation kit (Ambion). The amplification efficiency and template specificity of each of the primer pairs (listed in Table 2) were validated, and reaction mixtures were prepared as previously described (44). Quantitative real-time PCR (qRT-PCR) was performed using a one-step reaction with an ABI 7500-Fast sequence detection system (Applied Biosystems). Data were collected using ABI Sequence Detection software, version 1.2 (Applied Biosystems). All data were normalized to levels of *rpoA* and analyzed using the comparative cycle threshold (C_T) method (45). Target gene expression levels were compared by the relative quantification method (45). Statistical significance was determined by Student's *t* test.

Reverse transcriptase PCR (RT-PCR). SuperScript II reverse transcriptase (Invitrogen) and random primers were used to create cDNA from RNA samples. The cDNA was used for PCR with gene-specific primers (Table 2). Genomic DNA was used as a positive control, and a reaction without reverse transcriptase was used as a negative control.

FAS assay. Fluorescent actin staining (FAS) assays were performed as described previously (46). Briefly, overnight bacterial cultures were grown in LB broth at 37°C and then diluted 1:100 to infect HeLa cells. Infected HeLa cells were grown on coverslips for 6 h at 37°C with 5% CO_2. Subsequently, the coverslips were washed and fixed with formaldehyde, and then the membranes were permeabilized with 0.2% Triton-X and stained with fluorescein isothiocyanate (FITC)-labeled phalloidin to visualize actin. Bacteria and HeLa cell nuclei were stained with propidium iodide. AE lesions formed by each strain were enumerated for at least 400 HeLa cells in each experiment. Two independent experiments with three biological replicates of each condition were performed. Statistical significance was determined by Student's *t* test.

Secreted protein immunoblotting. Secreted proteins were collected as previously described (2). Secreted proteins from culture supernatants were separated from bacterial cells using centrifugation and filtration. SDS-PAGE and immunoblotting were performed as previously described (47). Samples were subjected to immunoblotting with rabbit polyclonal antiserum to EspA and visualized with enhanced chemiluminescence (Bio-Rad). Coomassie blue staining was used to visualize bovine serum albumin (BSA) loading controls. Expression of EspA was quantified from three replicate samples using ImageJ and normalized to BSA levels. Expression levels are shown relative to those of the WT. Three independent experiments were performed.

Purification of EtrB and QseA. The EtrB protein was fused with the maltose-binding protein (MBP) using a pMAL-C5x vector (NEB) with the restriction enzymes NcoI and SbfI (NEB) to create pDL02. *E. coli* strain BL21(DE3) containing pDL02 was grown at 37°C in LB broth with

TABLE 2 Primers used in this study

Primer name	Sequence	Primer use
etrB_GenComp_F	AAGCTTGTATTCTTTGGATTTTGCTTA	*etrB* complement
etrB_GenComp_R	CCATGGACTAGGCTTAATGAACTAGA	*etrB* complement
etrB_MalComp_F	CCTGCAGGTATAGTGCACACACCCATAC	*etrB*::MBP fusion
etrB_MalComp_R	CCATGGGAATGATGGGGGCCGAACTC	*etrB*::MBP fusion
etrB_Lux_F	GTTTAAACTAATTATATTTCATTATTATTTCATC	*etrB*::*lux* fusion
etrB_Lux_R	TACGTATTAACCCATTTTACGAGTTC	*etrB*::*lux* fusion
etrB_LR_F	TATTTTAGGAGAATTTGCAGGTGGAATGATGGGGGCCGAACTCGTAAAATTGTGTAGGCTGGAGCTGCTTC	*etrB* mutant
etrB_LR_R	CAAAATGAGCCTAAAGCCTCTTTTTTTTATATAGTGCACACACCCATACGTCATATGAATATCCTCCTTAG	*etrB* mutant
etrB_RT_F1	GGGCCGAACTCGTAAAATGG	qRT-PCR
etrB_RT_R1	ATACGCATCCTTTCGCACCT	qRT-PCR
ler_RT_F1	CGACCAGGTCTGCCC	qRT-PCR
ler_RT_R1	GCGCGGAACTCATC	qRT-PCR
grlA_RT_F1	CCGGTTGTTCCAGGACTTTC	qRT-PCR
grlA_RT_R1	TAAGCGCCTTGAGATTTTCATTT	qRT-PCR
escC_RT_F1	GCGTAAACTGGTCCGGTACGT	qRT-PCR
escC_RT_R1	TGCGGGTAGAGCTTTAAAGGCAAT	qRT-PCR
escV_RT_F1	TCGCCCCGTCCATTGA	qRT-PCR
escV_RT_R1	CGCTCCCGAGTGCAAAA	qRT-PCR
espA_RT_F1	TCAGAATCGCAGCCTGAAAA	qRT-PCR
espA_RT_R1	CGAAGGATGAGGTGGTTAAGCT	qRT-PCR
eae_RT_F1	GCTGGCCCTTGGTTTGATCA	qRT-PCR
eae_RT_R1	GCGGAGATGACTTCAGCACTT	qRT-PCR
rpoA_RT_F1	GCGCTCATCTTCTTCCGAAT	qRT-PCR
rpoA_RT_R1	CGCGGTCGTGGTTATCTG	qRT-PCR
ygeI_RT_F1	TAGCGAATGCAACGGGTGAT	qRT-PCR
ygeI_RT_R1	GACGCCATCCATGTTGAAACT	qRT-PCR
yqeK_RT_F1	ATGGACATTGAGTTTTCGCAGA	qRT-PCR
yqeK_RT_R1	CCCATGATGTTGTTTGCGTGA	qRT-PCR
ryeA_RT_F1	AGATGACGACGCCAGGTTTT	qRT-PCR
ryeA_RT_R1	ACCAGAACGGGCGGTTTTTA	qRT-PCR
tnaA_RT_F1	TGTACACCGAGTGCAGAACC	qRT-PCR
tnaA_RT_R1	CCGTCATACAGACCTACGGC	qRT-PCR
eivF_RT_F1	TTGTTTGCTGATGCCTTGCC	qRT-PCR
eivF_RT_R1	CGCTGCTCAGATAAGTGGCT	qRT-PCR
etrA_RT_F1	TGCAAGTCTTTTCCAGTGATGTC	qRT-PCR
etrA_RT_R1	CCAACGCAACTAAATCGCTGT	qRT-PCR
malK_RT_F1	CGCAATCGATCAAGTGCAGG	qRT-PCR
malK_RT_R1	GTCAGCGATATCACTCGGCA	qRT-PCR
nleA_RT_F1	TGTTGAAGGCTGGAAGTTTGTTT	qRT-PCR
nleA_RT_R1	CCGCTACAGGGCGATATGTT	qRT-PCR
Z4498_RT_F1	CTTGGCAAAAGTGGGCTCTT	qRT-PCR
Z4498_RT_R1	CCGCATCGTCAATACGGATA	qRT-PCR
stx2a _RT_F1	ACCCCACCGGGCAGTT	qRT-PCR
stx2a _RT_R1	GGTCAAAACGCGCCTGATA	qRT-PCR
etrB_prom_F1	TATTATTTCATCAATGTATTCTTT	EMSA
etrB_prom_R1	CGATTTAACCCATTTTACGA	EMSA
kan_EMSA_F1	CCGGAATTGCCAGCTGGGGCG	EMSA
kan_EMSA_R1	TCTTGTTCAATCATGCGAAACGATCC	EMSA
ler_emsaF	ATGCAATGAGATCTATCTTA	EMSA
ler_emsaR	AATATTTTAAGCTATTAGCG	EMSA
amp_emsaF	GGAATTCGAAAGGGCCTCGTGATACGC	EMSA
amp_emsaR	CGGGATCCGGTGAGCAAAAACAGGAAGG	EMSA
Z4175_R	GATAGCATAGGGAAGAACAG	RT-PCR
Z4177_F	TCACTGGCTCAGGTTTAATG	RT-PCR
Z4178_F	GGAATGTCCATATTGCATATC	RT-PCR
Z4176_F	TTACAACTCATAGCTGATGG	RT-PCR
Z4176_R	TTCCATACGCATCCTTTCGC	RT-PCR
etrB_PE_R	CGACTGTTTTCCTGCTTAAC	RT-PCR

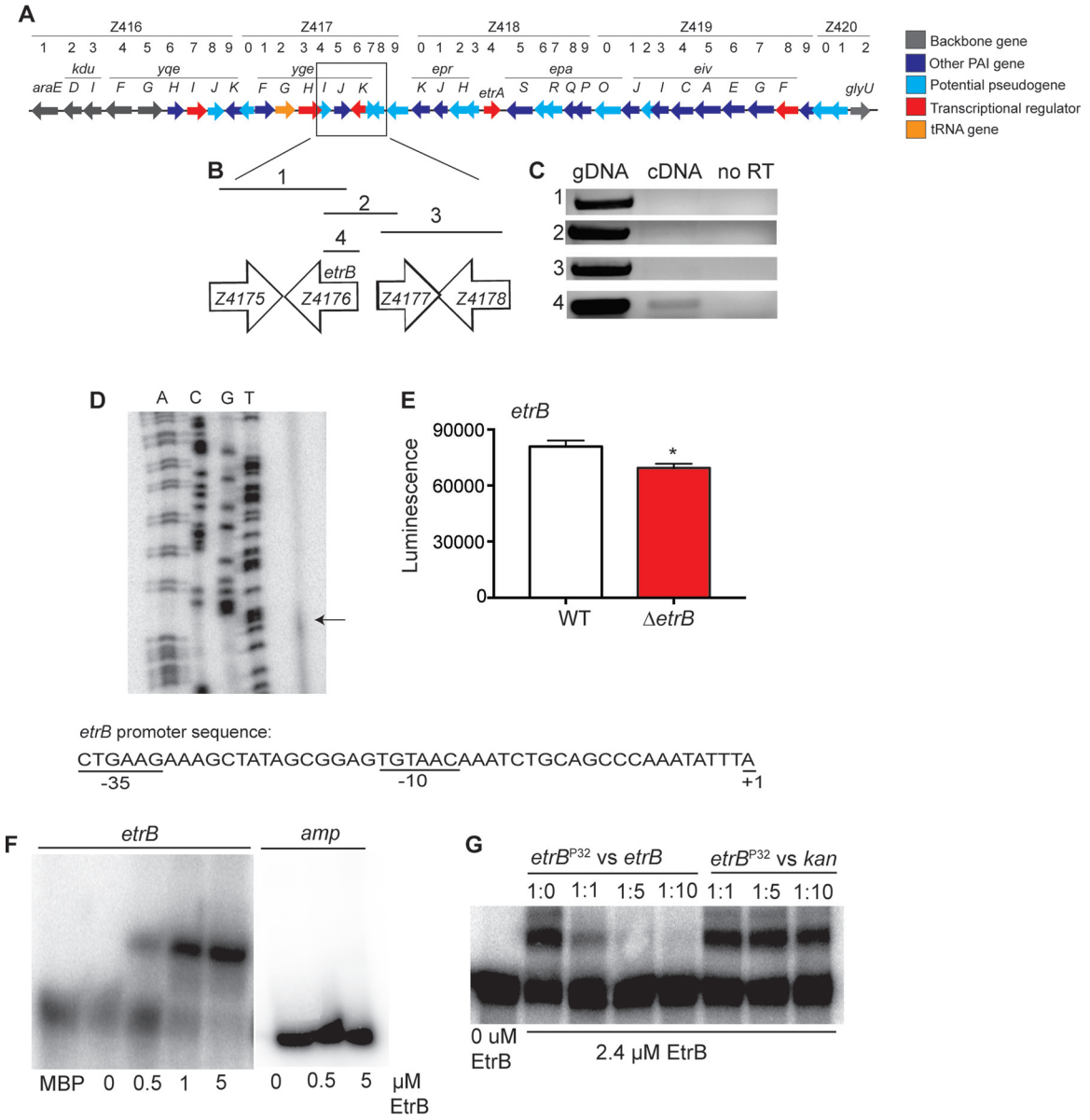

FIG 1 *etrB* expression and autoregulation. (A) Schematic representation of the ETT2 genetic locus in EHEC. PAI, pathogenicity island. (B) Inset of *etrB* and adjacent genes. Lines with numbers indicate regions amplified and correspond to PCRs shown in panel C. (C) RT-PCR of *etrB* and adjacent genes. Genomic DNA (gDNA) was used as a positive control, and a reaction without RT was used as a negative control. (D) Primer extension assay of *etrB*. The first four lanes show the *etrB* sequencing ladder. The arrow represents the transcription start site of *etrB*. The promoter sequence of *etrB* is shown with the predicted transcription start site and −10 and −35 regions. (E) Expression of *etrB* in WT 86-24 or the Δ*etrB* strain transformed with the *etrB*::*lux* reporter ($n = 3$; error bars represent the geometric means ± standard deviations). *, $P \leq 0.05$. (F) EMSA of the *etrB* promoter and *amp* negative-control promoter region with MBP or EtrB::MBP. (G) Competition assays with EtrB. The assay was performed with increasing amounts of the unlabeled *etrB* probe or the unlabeled *kan* probe as a negative control.

glucose (0.2% final concentration) and ampicillin (100 μg/ml) to an OD_{600} of 0.5. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.3 M, and protein expression was induced overnight at 16°C. Cells were then pelleted by centrifugation at 4,000 × *g* for 10 min and resuspended in column buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA). The cells were lysed with an EmulsiFlex instrument. The lysed cells were centrifuged at 4°C, and the supernatant was loaded onto a gravity column (Qiagen) with amylose resin. The column was washed with column buffer, and protein was eluted from the column using column buffer containing 10 mM maltose. Fractions containing purified EtrB were confirmed by SDS-PAGE and Western analysis. The His-tagged QseA protein from plasmid pMK08 was purified as previously described (39). Briefly, the *E. coli* strain BL21(DE3) containing pMK08 was grown to an OD_{600} of 0.5 and then induced with 0.4 M IPTG for 3 h. Cells were lysed as described above, and purification was performed using gravity columns (Qiagen) with nickel beads. The column was washed with nickel wash buffer (50 mM $NaPO_4$, 300 mM NaCl, 20 mM imidazole), and protein was eluted using nickel wash buffer containing 250 mM imidazole.

EMSAs. Electrophoretic mobility shift assays (EMSAs) were performed using the purified EtrB-MBP, QseA-His, and PCR-amplified DNA probes (Table 2), as previously described (48). DNA probes were end labeled using T4 polynucleotide kinase (NEB) with [^{32}P]ATP (Perkin-Elmer) (47). End-labeled probes were purified using Invitrogen NucAway spin columns. EMSAs were performed by adding increasing amounts of puri-

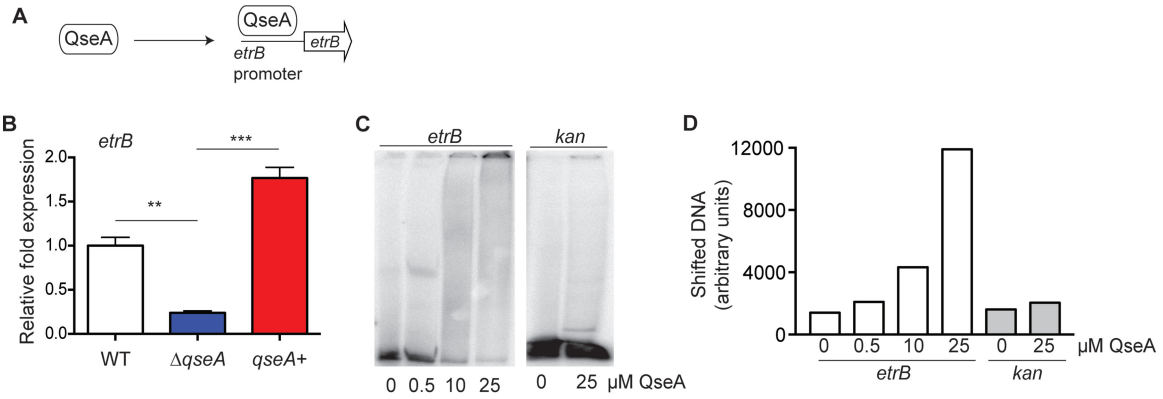

FIG 2 QseA activates *etrB* expression. (A) Schematic of model being tested. (B) qRT-PCR of *etrB* expression in WT 86-24, the Δ*qseA* strain, and the *qseA* complemented strain (*qseA*$^+$) ($n = 3$; error bars represent the geometric means ± standard deviations). **, $P \leq 0.005$; ***, $P \leq 0.0005$. (C) EMSA of the *etrB* promoter and the *kan* negative-control promoter regions with QseA protein. (D) Quantification of shifted *etrB* or *kan* DNA depicted in panel C.

fied QseA or EtrB protein to end-labeled probe in binding solution [500 μg/ml BSA, 50 ng of poly(dI-dC), 60 mM HEPES, pH 7.5, 5 mM EDTA, 3 mM dithiothreitol (DTT), 300 mM KCl, and 25 mM $MgCl_2$] and incubated at room temperature for 25 min. A 1% Ficoll solution was added to the reaction mixtures immediately before the samples were loaded on the gel. The reaction mixtures were electrophoresed on a 6% polyacrylamide gel for approximately 6 h at 150 V, dried, and imaged with a PhosphorImager (Molecular Dynamics). Bands were quantified using ImageQuant software.

Primer extension. Primer extension analysis was performed as previously described (39). Briefly, primer etrB_PE_R (Table 2) was end labeled as described above. A total of 40 μg of RNA, isolated from strain 86-24, was used to generate cDNA using a Primer Extension System with an avian myeloblastosis virus (AMV) reverse transcriptase kit (Promega). The resultant cDNA was precipitated, electrophoresed on a 6% polyacrylamide-urea gel next to a sequencing reaction (Affymetrix). Amplified genomic DNA from strain 86-24 was used to generate a sequencing ladder using primers etrB_prom_F1 and etrB_PE_R for the *etrB* promoter.

Microarray. Affymetrix *E. coli* Genome 2.0 gene arrays were used to compare gene expression of strain 86-24 to that of DL01 (Δ*etrB*) as previously described (33). The RNA processing, labeling, hybridization, and slide-scanning procedures were performed as described in the Affymetrix *GeneChip Expression Analysis Technical Manual* (49). Data analyses from the array were performed as previously described (50). The Affymetrix GeneChip Command Console (AGCC) software was used to obtain the output from scanning a single replicate of the Affymetrix GeneChip *E. coli* Genome 2.0 array for each of the biological conditions, according to the manufacturer's instructions. Data were normalized using robust multiarray analyses (RMA), and the resulting data were compared to determine genes whose expression was increased or decreased in response to the presence of *etrB*.

RESULTS

Identification and characterization of *etrB*. The gene *etrB* (*ygeK*, MG1655 genome; *Z4176*, EDL933 genome; *ECs712*, Sakai genome [24, 25]) is carried in the ETT2 pathogenicity island (Fig. 1A) and is predicted to encode a 16-kDa protein that shares homology to NarL-type transcription factors, including the *Salmonella* regulatory protein SsrB (26). The *etrB* open reading frame (ORF) is present in several *E. coli* strains; however, bioinformatics analyses indicate that *etrB* is a pseudogene in nonpathogenic *E. coli* K-12 strains (26). In pathogenic EHEC strains, the potential functionality of EtrB is less clear. The Sakai and EDL933 annotated genome sequences predict different ORFs, with *etrB* encoding a 663-bp gene in Sakai or a 447-bp gene in EDL933 (24, 25). We studied EHEC pathogenesis using the strain 86-24, which was isolated from a patient suffering hemorrhagic colitis (51) and has been used in several EHEC animal models (52–59). To begin to characterize *etrB* in strain 86-24, we performed RT-PCR to determine whether *etrB* was expressed as part of a transcript with the adjacent up- or downstream genes (depicted in Fig. 1B). For this, we used cDNA synthesized from RNA that was purified from WT EHEC grown statically for 6 h in DMEM. No PCR products were obtained in reactions that included primers specific for the flanking genes (Fig. 1B and C); however, a PCR product was visible when primers specific for *etrB* were used in the reaction (Fig. 1C). These findings indicate that *etrB* is expressed in EHEC strain 86-24 but that it is not cotranscribed with the immediate up- or downstream genes.

To further characterize *etrB* expression and map the promoter region, we performed primer extension analyses. For this, we designed a primer approximately 50 bp downstream from the *etrB* translational start codon. Then, primer extension analysis was performed using cDNA synthesized from RNA. The primer extension results revealed one transcriptional start site in the *etrB* promoter (Fig. 1D), which we mapped to approximately 44 bp upstream of the translational start site. These data are consistent with the annotation of the EDL933 genome. These data suggest a −10 sequence, TGTAAC, and a −35 sequence, CTGAAG, which contain two and three mismatches from the σ^{70} consensus sequences (TATAAT and TTGACA), respectively, and which are separated by 15 nucleotides (Fig. 1D).

To begin to characterize EtrB expression, we examined whether EtrB autoregulates transcription. For this, we generated a deletion of *etrB*. The deletion of *etrB* did not impact the EHEC growth rate under our experimental conditions, as the wild-type (WT) and Δ*etrB* strains reached similar ODs after 6 h of static growth in DMEM (WT OD_{600}, 0.841 ± 0.008; Δ*etrB* strain OD_{600}, 0.842 ± 0.004). Then, we transformed the WT and the Δ*etrB* strains with a plasmid containing the *etrB* promoter fused to the *luxCDABE* gene cluster that encodes bacterial luciferase (60). Expression of *etrB*::*lux* was significantly decreased in the Δ*etrB* strain compared to that in the WT (Fig. 1E), indicating that EtrB positively autoregulates expression.

Proteins belonging to the NarL family bind DNA to regulate

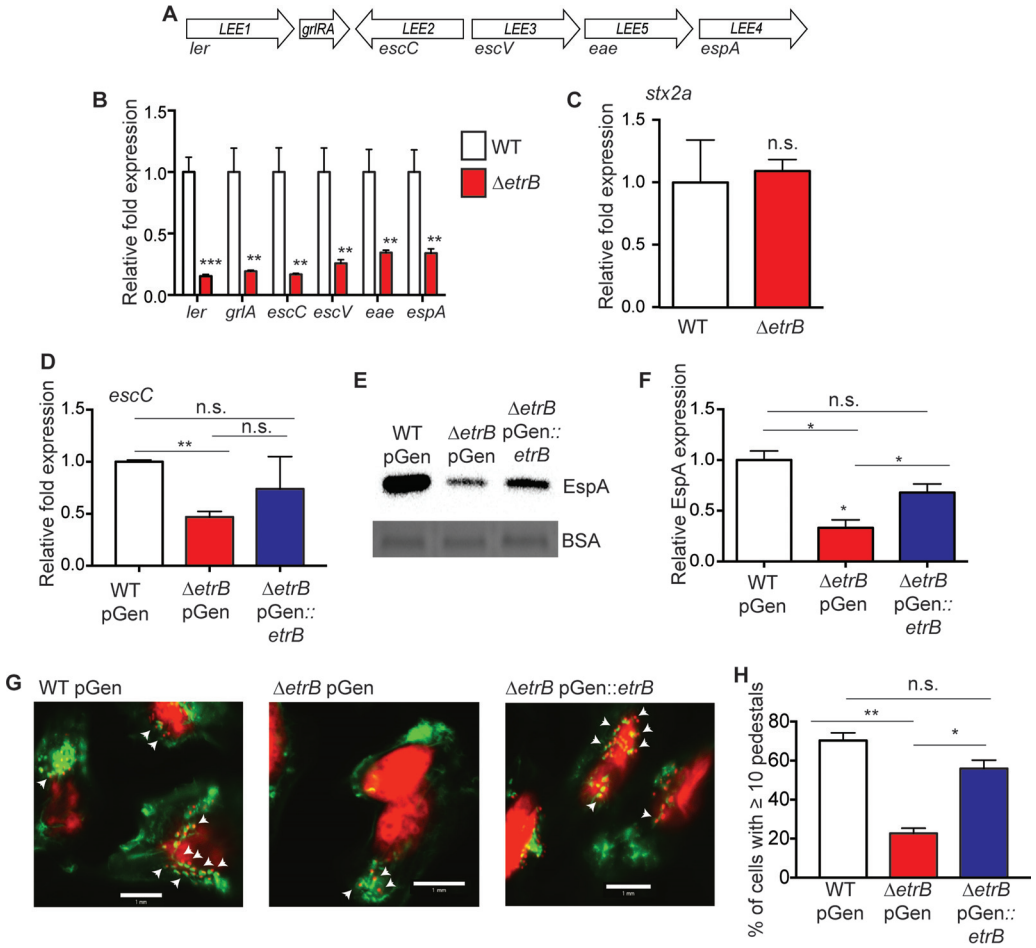

FIG 3 EtrB activates expression of the LEE. (A) Schematic of the LEE pathogenicity island. (B) qRT-PCR of *ler*, *grlA*, *escC*, *escV*, *eae*, and *espA* in WT 86-24 and the Δ*etrB* strains. (C) Expression of stx_{2a} by qRT-PCR in WT 86-24 and the Δ*etrB* strains. (D) Expression of *escC* by as determined by qRT-PCR in the WT 86-24 strain transformed with an empty vector control, the Δ*etrB* strain transformed with an empty vector control, and the Δ*etrB* strain complemented with *etrB*. (E) Representative Western blot of the LEE-encoded EspA secreted protein in WT 86-24 transformed with an empty vector control, the Δ*etrB* strain transformed with an empty vector control, and the Δ*etrB* strain complemented with *etrB*. Bovine serum albumin (BSA) is shown as a loading control. (F) Quantification of EspA expression from three independent assays from EHEC strains as described for panel E. (G) FAS assay with WT 86-24 transformed with an empty vector control, the Δ*etrB* strain transformed with an empty vector control, and the Δ*etrB* strain complemented with *etrB*. HeLa nuclei and bacteria were stained red with propidium iodide, and HeLa cell actin cytoskeleton was stained green with FITC-phalloidin. AE lesions are observed as punctate green structures associated with bacterial cells and are indicated by arrowheads. Scale bar, 1 mm. (H) Percentage of HeLa cells with greater than 10 AE lesions. Statistical significance is shown relative to results with the WT 86-24 strain unless otherwise indicated. *, $P \leq 0.05$; **, $P \leq 0.005$; ***, $P \leq 0.0005$; ns, not significant.

transcription (61). Therefore, we performed EMSAs to investigate whether EtrB binds its own promoter to regulate transcription. For this, we constructed a plasmid that expresses a fusion protein in which the C terminus of MBP was fused to the N terminus of EtrB. EMSAs indicated that EtrB directly binds its promoter to regulate expression (Fig. 1F). To confirm specificity of binding, we performed EMSAs with purified MBP alone as well as competition EMSAs. Purified MBP did not bind the *etrB* promoter (Fig. 1F). Moreover, EtrB binding was outcompeted by the addition of unlabeled *etrB* probe, whereas no competition was observed when increasing amounts of cold *kan* probe were added (Fig. 1G). Altogether, these findings indicate that *etrB* is expressed and encodes a functional protein that controls its own expression. Accordingly, we renamed the *ygeK* gene *etrB* (ETT2 transcriptional regulator B), according to the nomenclature of Zhang et al. (27).

We originally identified *etrB* in a microarray study as being activated by QseA (39). Here, we confirmed that QseA influences *etrB* transcription and tested whether this was through direct interaction (Fig. 2A). First, we performed qRT-PCR analyses using RNA extracted from WT, the Δ*qseA*, or the *qseA* (*qseA*$^+$) complemented strain. Transcription of *etrB* was significantly decreased in the Δ*qseA* strain compared to the level in the WT, and complementation of the Δ*qseA* strain restored *etrB* expression to WT levels (Fig. 2B). To determine whether QseA influenced *etrB* expression directly, we performed EMSAs. We observed a shift with the addition of purified QseA to radiolabeled *etrB* promoter DNA; however, no shift was observed using the negative-control *kan* DNA (Fig. 2C). Quantification of radiolabeled DNA confirmed that the amount of shifted *etrB* DNA correlated with increasing amounts of QseA added to the reaction mixture (Fig. 2D). Collectively, these data indicate that *etrB* is a direct target of QseA regulation.

EtrB activates LEE transcription. To determine whether EtrB plays a role in directing virulence gene expression in EHEC, we

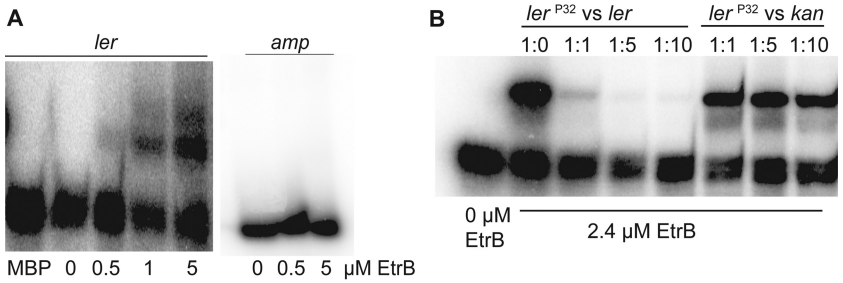

FIG 4 EtrB directly binds to the *ler* promoter region to activate LEE expression. (A) EMSA of the *ler* promoter and *amp* negative-control promoter region with MBP or EtrB::MBP. (B) Competition assays with EtrB. The assay was performed with increasing amounts of the unlabeled *ler* probe or the unlabeled *kan* probe as a negative control.

performed qRT-PCR and measured expression of one gene in each LEE operon (Fig. 3A), as well as expression of the stx_{2a} gene, which encodes Shiga toxin. Transcription of *ler*, located in *LEE1*, as well as transcription of *grlA* and operons *LEE2* to *LEE5*, was significantly decreased in the Δ*etrB* strain compared to that in the WT (Fig. 3B); however, no differences in stx_{2a} expression levels were measured (Fig. 3C). Additionally, *trans* complementation with *etrB* on a low-copy-number plasmid nearly restored LEE mRNA and protein to WT levels (Fig. 3D to F).

To functionally test the impact of EtrB on LEE expression, we assessed AE lesion formation. For this, we performed an FAS assay (46) and determined the number of pedestals formed on epithelial cells by WT EHEC, the Δ*etrB* strain, and the *etrB* complemented strain. In agreement with the LEE expression data, we measured significantly fewer pedestals when HeLa cells were infected with the Δ*etrB* strain than when cells were infected with WT EHEC, and the *etrB* plasmid was able to complement the Δ*etrB* strain (Fig. 3G and H).

To determine whether EtrB directly regulates LEE expression, we performed EMSAs. For these experiments, we generated a probe containing the entire *ler* regulatory region. EtrB shifted the radiolabeled *ler* DNA but not the negative-control *amp* promoter (Fig. 4A). To ensure specificity of binding, we performed EMSAs using MBP alone as well as competition EMSAs, as described for the *etrB* promoter. In these assays, no shift was observed with MBP alone (Fig. 4A). Additionally, the unlabeled *ler* probe competed for EtrB binding at a ratio of 1:1 (labeled probe to unlabeled probe) (Fig. 4B); however, the negative-control *kan* probe exhibited no competition for binding (Fig. 4B). These findings indicate a specific and direct interaction between EtrB and the *ler* (*LEE1*) regulatory region.

EtrB transcriptome analyses. To investigate the global role of EtrB in EHEC gene regulation, we performed transcriptome analyses using the Affymetrix *E. coli* Genome 2.0 gene microarrays. These data revealed that EtrB functions to both positively and negatively influence gene expression in EHEC. For example, 46 gene probe sets were decreased and 70 probe sets were increased in the Δ*etrB* strain compared to levels in the WT (using a ≥2-fold change in expression as the cutoff for differentially regulated probes) (see Table S1 in the supplemental material). Genes regulated by EtrB included virulence factors, such as the LEE genes and non-LEE-encoded effectors, adhesins and ETT2-located genes, genes important for metabolism, and the noncoding RNA, RyeA/SraC.

We confirmed a subset of transcripts that were differentially regulated in the array. NleA is an effector that is encoded outside the LEE but which is secreted through the LEE-encoded T3SS. In EHEC, NleA has diverse functions and has been shown to disrupt tight junctions of epithelial cells, inhibit protein secretion, and modulate the host immune response (21, 62, 63). Moreover, an *nleA* deletion strain is attenuated during murine infection, highlighting its importance to EHEC pathogenesis (21). EtrB positively influences *nleA* expression as *nleA* transcription was decreased in the Δ*etrB* strain compared to that in the WT (Fig. 5A). NleA expression and secretion are at least partly dependent on Ler (64); thus, the decrease in NleA in the Δ*etrB* strain may be an indirect result of EtrB-dependent regulation of LEE expression.

In addition to activating expression of virulence factors, EtrB positively influences transcription of genes involved in metabolism and posttranscriptional gene expression. For example, genes involved in tryptophan and maltose utilization were decreased in the Δ*etrB* strain (Fig. 5B and C). Additionally, transcript levels of the sRNA RyeA/SraC were decreased in the Δ*etrB* strain compared to the level in the WT (Fig. 5D). RyeA/SraC is present as a 270-bp RNA during exponential growth and is processed to a shorter 150-bp RNA during stationary phase (65–67). These findings sug-

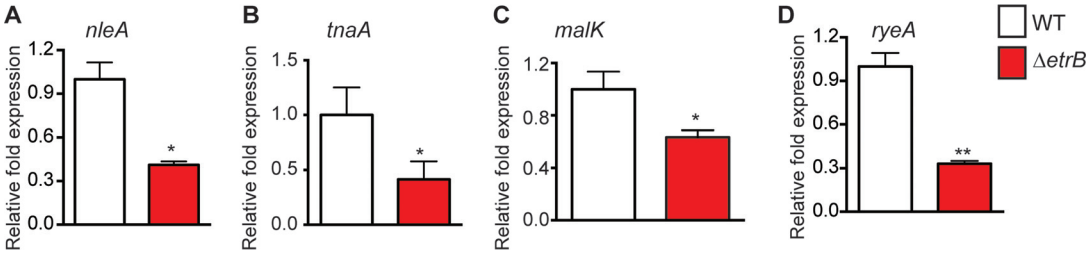

FIG 5 EtrB-positive targets of regulation. qRT-PCR of indicated genes (described in the main text) was performed in WT 86-24 and the Δ*etrB* strain ($n = 3$; error bars represent the geometric means ± standard deviations). Statistical significance is shown relative to results with the WT 86-24 strain. *, $P \leq 0.05$; **, $P \leq 0.005$.

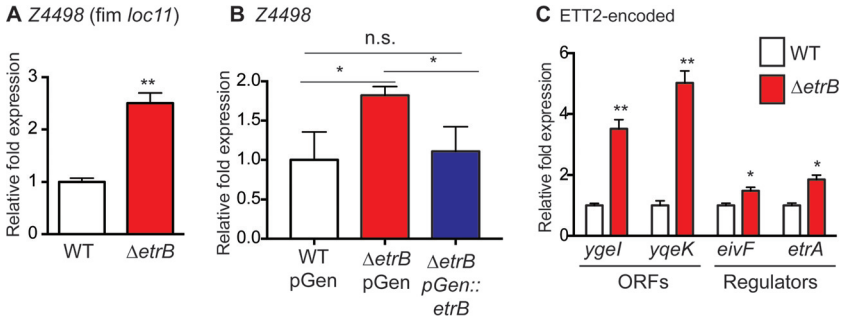

FIG 6 EtrB negatively regulates transcription of fimbrial locus 11 and genes located in the ETT2. (A) qRT-PCR of *Z4498* in WT 86-24 and the Δ*etrB* strain. (B) qRT-PCR of *Z4498* in the WT 86-24 strain transformed with an empty vector control, the Δ*etrB* strain transformed with an empty vector control, and the Δ*etrB* strain complemented with *etrB* ($\Delta n = 3$; error bars represent the geometric means ± standard deviations). (C) qRT-PCR of indicated genes in WT 86-24 and the Δ*etrB* strain. Statistical significance is shown relative to results with WT 86-24 unless otherwise indicated. *, $P \leq 0.05$; **, $P \leq 0.005$; ns, not significant.

gest that RyeA/SraC plays a role during the stress response; however, the biological role of this sRNA is not known.

EHEC encodes 14 to 16 fimbrial loci which mediate attachment to epithelial cells. The fimbrial locus 11 (*loc11*) belongs to the chaperone-usher family of adhesins. These types of fimbriae typically are composed of a chaperone, an usher, and a major fimbrial subunit and may also include additional minor subunits (68). The microarray data indicated that EtrB represses expression of *loc11*. This locus contains seven ORFs, all of which have been shown to be expressed and cotranscribed (69); therefore, we confirmed the microarray data by measuring expression of the first gene in this operon, *Z4498*, which is predicted to encode the major fimbrial subunit. Expression of *Z4498* was significantly increased in the Δ*etrB* strain compared to the level in WT EHEC (Fig. 6A), and complementation restored expression to WT levels (Fig. 6B). Collectively, these data suggest that EtrB is important for coordinating expression of fimbrial- and LEE-mediated adherence.

The ETT2 pathogenicity island carries 35 ORFs that are predicted to encode effectors, components of a T3SS, and transcription factors and to harbor pseudogenes (24–26). The microarray data indicated that EtrB represses expression of ORFs located in the ETT2 pathogenicity island. For example, the expression levels of the ORFs *ygeI* and *yqeK* were significantly increased in the Δ*etrB* strain compared to expression in the WT (Fig. 6C). Additionally, expression of the ETT2-located regulators *eivF* and *etrA* was increased in the Δ*etrB* strain (Fig. 6C).

DISCUSSION

The ETT2 locus is present in the majority of *E. coli* strains; however, many of the ETT2 gene clusters carry mutations and deletions, suggesting that the ETT2 T3SS is not functional (26). Despite this fact, deletions of ETT2-encoded regulatory or structural proteins impact virulence in EHEC as well as in meningitis-causing *E. coli* strains (27, 70, 71). However, no biochemical evidence of how ETT2-located genes affect pathogenesis has been reported. EtrB belongs to the NarL family of transcription factors, which can function independently or function as part of a two-component system (TCS) (72, 73). The *etrB* gene is not located adjacent to a putative histidine kinase, which is typical for a cognate TCS (74). A previous study predicted that EtrB might function as an orphan response regulator and demonstrated that purified EtrB could be phosphorylated *in vitro* (75); however, the physiological relevance of this was not further examined. Our studies indicate that EtrB is able to bind target DNA in the absence of phosphorylation (Fig. 1 and 4), suggesting that EtrB acts as an independent transcription factor.

We mapped the EtrB regulon, and our data revealed that EtrB plays a broad role in EHEC gene expression, affecting expression of genes important for virulence, metabolism, and posttranscriptional gene expression (Fig. 3 to 6 and summarized in 7). Specifically, EtrB activates LEE expression through direct interaction with the *LEE1* regulatory region (Fig. 4). This is distinct from the regulatory influence of EtrA and EivF, which repress LEE expression (27). Interestingly, *etrA* and *eivF* expression was increased in the Δ*etrB* strain (Fig. 6C), suggesting that EtrB functions to promote AE lesion formation not only by direct interaction with the *LEE1* regulatory region but also by repressing expression of negative regulators of the LEE.

Finally, we provide biochemical evidence showing that *etrB* is a direct regulatory target of QseA. Therefore, we propose that QseA and EtrB function in a coherent feed-forward loop (FFL) (76). This FFL has important implications for the regulatory dynamics of LEE expression. For example, the coordination of multiple reg-

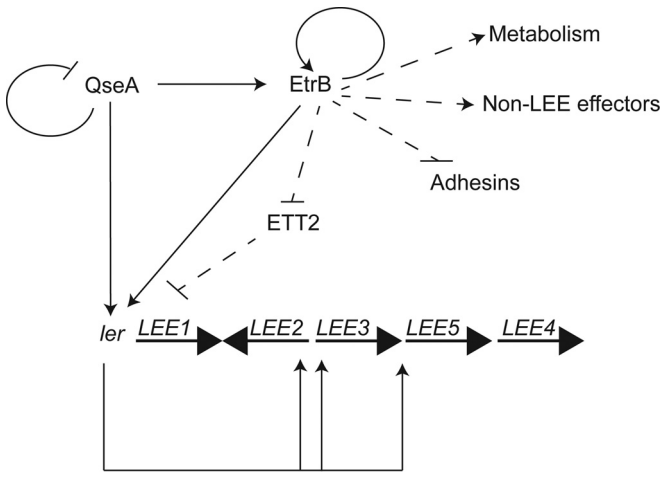

FIG 7 Model of the EtrB regulatory circuit. Lines with arrows indicate positive regulation; lines with bars indicate negative regulation. Solid lines represent direct interactions that have been biochemically defined; dashed lines represent interactions that occur indirectly or that have not been shown to bind biochemically to the target.

ulators might act to amplify environmental cues and promote AE lesion formation. Additionally, a previous study demonstrated that QseA negatively regulates its own transcription (77). In this model, as QseA levels decrease due to autoregulation, EtrB could still act to promote LEE expression, thereby prolonging expression to ensure efficient colonization of the gastrointestinal tract. Overall, this study has identified EtrB as an important regulator of gene expression in EHEC and provides a mechanistic understanding as to how ETT2-encoded regulators influence bacterial pathogenesis.

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