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CsrA and TnaB Coregulate Tryptophanase Activity To Promote Exotoxin-Induced Killing of *Caenorhabditis elegans* by Enteropathogenic *Escherichia coli*[▽]

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Enteropathogenic *Escherichia coli* (EPEC) requires the *tnaA*-encoded enzyme tryptophanase and its substrate tryptophan to synthesize diffusible exotoxins that kill the nematode *Caenorhabditis elegans*. Here, we demonstrate that the RNA-binding protein CsrA and the tryptophan permease TnaB coregulate tryptophanase activity, through mutually exclusive pathways, to stimulate toxin-mediated paralysis and killing of *C. elegans*.

Enteropathogenic *Escherichia coli* (EPEC) belongs to the attaching and effacing (A/E) family of pathogens, the other members of which include enterohemorrhagic *Escherichia coli* (EHEC) and *Citrobacter rodentium* (8, 17, 46, 56, 58, 64, 72). Upon infection, A/E pathogens bind to intestinal epithelia and destroy the cellular microvilli in their vicinity (8, 17, 58). Subsequently, the bacteria recruit several host factors that cooperate to promote the biogenesis of actin-filled membranous protrusions, termed "pedestals," beneath adherent bacteria (17, 25, 46, 49, 58, 64). Pedestal formation is accompanied by severe diarrhea, which results in significant morbidity and mortality worldwide (17, 34, 74).

Penetrance of the A/E pathomorphology requires the pathogenicity island (PAI), locus of enterocyte effacement (LEE) that encodes for the regulators, structural components of a type III secretion system (T3SS), and several of its secreted effector molecules (8, 18, 20, 25, 28, 46, 47, 58, 60, 73, 91). The *LEE1*-encoded master regulator (Ler) orchestrates the coordinated transcription from the other LEE operons to promote morphogenesis of the T3SS that forms a continuous conduit between the bacterial and the host cytoplasm (5, 15, 25, 28, 31, 47, 58, 60, 78). Subsequently, effectors, including the translocated intimin receptor (Tir), are trafficked into the host (18, 46, 47). Tir integrates into the host plasma membrane, where it serves as a receptor for its ligand, the adhesin intimin, located on the outer bacterial membrane (47). Tir-intimin interactions initiate a signal transduction cascade that leads to actin polymerization and pedestals (10, 41, 45, 47, 83).

A significant obstacle in elucidating the pathobiology of EPEC infections is that this bacterium is a human pathogen that neither colonizes nor causes disease in mice (62). Over the past decade, the capacity of bacterial pathogens, including EPEC, to kill the nematode *Caenorhabditis elegans* has been utilized to identify virulence determinants in the bacteria that may be relevant to pathogenesis in mammalian systems (61, 75). Its small size, rapid generation time, large brood size, amenability to genetic manipulation, and high degree of homology to humans and other mammals make *C. elegans* a useful experimental system with which to study bacterial toxins or infection (1, 61, 75, 76).

The morbidity and mortality in *C. elegans* caused by noxious microbes can be classified into two broad categories on the basis of whether the pathogen makes contact with the worm (1, 61, 75, 76). Contact-dependent killing usually involves the detrimental colonization of the worm in the form of a biofilm (e.g., *Yersinia pestis*) (21, 84), an invasive infection (e.g., *Streptomyces albireticuli*) (67), or accumulation within the intestine (e.g., EPEC) (59). The death of the nematode, as a consequence of colonization, typically occurs over several days and is referred to as "slow killing" (75). In contrast, contact-independent killing is mediated through structurally and functionally unrelated exotoxins that are secreted by diverse pathogens, including EPEC (2, 3), *Pseudomonas aeruginosa* (32, 57), and *Burkholderia cenocepacia* (51), among others, that lead to toxicity in the nematode (75). Intoxication of the worms is a relatively rapid pathophysiological process occurring over a period of hours and is referred to as "fast killing" (75). The utility of *C. elegans* as a surrogate host for mimicking bacterial infections has been repetitively substantiated by numerous studies in which novel virulence factors that were identified employing worm-based screens were subsequently shown to modulate virulence in mammalian systems (2, 33, 57, 75, 85). In reciprocal studies, virulence factors originally implicated in mammalian and plant pathogenesis were demonstrated to coregulate pathogenesis in worms (75, 76, 86).

EPEC is capable of killing *C. elegans* by contact-dependent and -independent means (2, 59). On minimal nematode growth medium (NGM), EPEC kills *C. elegans* over a period of several days by colonizing its intestinal tract (59). However, no virulence factors that contribute to the contact-dependent killing of the worm have thus far been discovered (59). Moreover, none of the virulence determinants previously implicated in mammalian pathogenesis were necessary for nematocidal activity (59). In contrast, on nutritionally rich medium (Luria-Bertani [LB] or *E. coli* direct agar [ECD]) supplemented with tryptophan, EPEC synthesizes diffusible exotoxins that lead to rapid paralysis and subsequent death of the nematode within a

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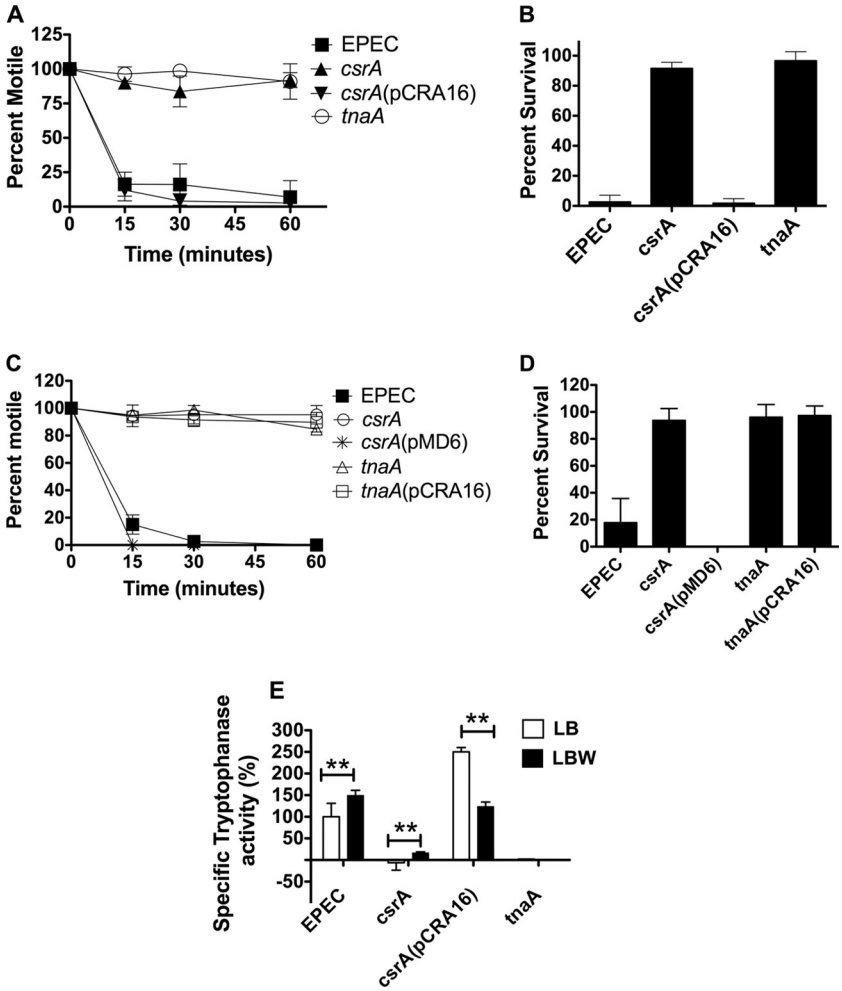

FIG. 1. (A to E) *csrA* regulates tryptophanase activity to promote toxin-mediated killing of *C. elegans* by EPEC. (A and B) Young adult worms were exposed to confluent lawns of EPEC, *csrA* mutant, *csrA* mutant complemented with a functional *csrA* allele expressed from a multicopy plasmid [*csrA* (pCRA16)], and *tnaA* mutant and monitored for paralysis (A) and killing (B) on LB agar supplemented with tryptophan (LBW). Worms were considered paralyzed if they failed to traverse an entire body length on prodding. Worm mortality was assayed by transferring the pathogen-exposed worms onto NGM plates containing nonpathogenic *E. coli* OP50 and assaying for motility 24 h later. Error bars indicate standard deviations of results from at least three independent experiments, with each employing at least two biological replicates. A one-way analysis of variance (ANOVA) was used to assess statistical significance. A P value cutoff of <0.05 was considered statistically significant. The calculated P values for both the paralysis and killing assays were <0.02. (C and D) Paralysis (C) and killing (D) of the nematodes were assayed in the presence of EPEC, *csrA* mutant, *csrA* mutant overexpressing *tnaA* [*csrA* (pMD6)], *tnaA* mutant, and *tnaA* mutant overexpressing *csrA* [*tnaA* (pCRA16)] essentially as described above. A one-way ANOVA was used to assess statistical significance. The calculated P values for both the paralysis and killing assays were <0.02. (E) Tryptophanase activity was measured from lysates of bacteria cultivated on agar plates. The rate of hydrolysis of SOPC, a chromogenic tryptophan analogue, to ONTP was measured as described previously (2). Error bars indicate standard deviations of results from at least two independent experiments, each with at least three replicates. The unpaired Student t test was employed to assay for statistical significance between the indicated samples. A P value cutoff of <0.01 was considered statistically significant. **, $P < 0.01$.

few hours (2, 3). Exotoxin-induced lethality requires the bacterial enzyme tryptophanase. Subsequently, it was shown that tryptophanase regulates the LEE in both EPEC and EHEC and consequently influences pedestal formation and mammalian pathogenesis (2, 42). However, other than tryptophanase, the EPEC-*C. elegans* pathosystem has not been exploited to identify additional virulence determinants that may contribute to morbidity in mammals.

In a previous study, we reported that the RNA-binding protein, CsrA, is necessary for EPEC to form pedestals on mammalian cells (7, 8). CsrA and its ortholog, RsmA, recognize AGGA/ANGGA tracts in the 5′-untranslated leader segments of transcripts and modulate mRNA stability and/or translation (7, 8, 26, 68). The relaxed sequence specificity of CsrA/RsmA enables this posttranscriptional regulator to modulate a panoply of physiological traits, such as carbon homeostasis (4, 27, 68–70), peptide uptake (27), biofilm formation (44, 88), motility (7, 14, 52, 90, 92), quorum sensing (19, 55), colicin biosynthesis (93), and virulence (7, 13, 19, 30, 40, 48, 55).

Here, we have evaluated the role of CsrA in the toxin-mediated killing of *C. elegans.* Bioassays employing worms were conducted on LB agar plates containing or lacking tryptophan essentially as described previously, with the modification that tryptophan was added to a final concentration of 1

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype/phenotype	Reference or source
Strains		
EPEC	Prototypical EPEC 2348/69 serotype O127:H6	Jim Kaper
EPEC *csrA*	EPEC 2348/69 Ω*csrA*::*cat*/Cm^r	7
EPEC *tnaA*	EPEC 2348/69 Δ*tnaA*::*cat*/Cm^r	2
EPEC *csrA* (pCRA16)	EPEC 2348/69 Ω*csrA*::*cat* transformed with the plasmid pCRA16/Cm^r Tc^r	This study
EPEC *csrA* (pMD6)	EPEC 2348/69 Ω*csrA*::*cat* transformed with the plasmid pMD6/Cm^r Ap^r	This study
EPEC *tnaA* (pCRA16)	EPEC 2348/69 Δ*tnaA*::*cat* transformed with the plasmid pCRA16/Cm^r Tc^r	This study
EPEC *tnaB*	EPEC 2348/69 Ω*tnaB*::Tn*5*-*kan*/Km^r	This study
EPEC *tnaB* (ptnaB)	EPEC 2348/69 Ω*tnaB*::Tn*5*-*kan* transformed with the plasmid ptnaB/Km^r Ap^r	This study
EPEC *mtr*	EPEC 2348/69 Δ*mtr*::*cat*/Cm^r	This study
EPEC *aroP*	EPEC 2348/69 Δ*aroP*::*cat*/Cm^r	This study
Plasmids		
pKD3	pANTSγ-(FRT-*cat*-FRT) R6Kγ*oriV*/Ap^r Cm^r	22
pCRA16	pBR322-Ω*bla*::(P_{csrA} -*csrA*$^+_{K\text{-}12}$)/Tc^r	82
pMD6	pBR322-(P_{tnaCAB} -*tnaCA*$_{K\text{-}12}$)/Ap^r	23
ptnaB	An EcoRI-PstI-restricted amplicon containing the *tnaB* ORF from EPEC 2348/69 cloned downstream of the P_{araBAD} promoter of the identically restricted plasmid, pBAD24/Ap^r	This study

mg/ml, and 200 μl of the overnight inoculum was seeded onto plates (2). Disruption of *csrA* abolished the ability of EPEC to paralyze (Fig. 1A) and kill (Fig. 1B) *C. elegans*. The *csrA* mutant regained its pathogenicity when complemented in *trans* with the plasmid pCRA16 that expresses *csrA* under its native promoters (Fig. 1A and B) (Table 1) (89).

The observation that disruption of *csrA* genocopies the effect of deleting *tnaA* (Fig. 1) suggested that the two genes might constitute components of the same regulatory pathway. In *E. coli*, *tnaA* is the central gene within a tricistronic operon that includes the upstream regulatory gene *tnaC* and the downstream structural gene *tnaB* (23, 24, 36). *tnaC* encodes a *cis*-acting regulatory peptide that governs the expression of *tnaA* and *tnaB* in response to tryptophan accumulation (37, 80). *tnaA* encodes for the catabolic enzyme tryptophanase, which catalyzes the hydrolysis of tryptophan into indole, pyruvate, and ammonia, whereas *tnaB* specifies a low-affinity tryptophan permease that facilitates the import of tryptophan into the bacterium (23, 54, 71, 77, 94). To elucidate the regulatory hierarchy of *csrA* and *tnaA*, each gene was expressed from a multicopy plasmid in the mutant background of the other. Whereas multicopy expression of *csrA* failed to restore virulence to the *tnaA* mutant, overexpression of *tnaA*, from the medium-copy-number plasmid pMD6 (Table 1), suppressed the attenuated phenotype of the *csrA* mutant and restored its ability to paralyze (Fig. 1C) and kill (Fig. 1D) *C. elegans*. The observation that increased expression of *tnaA* circumvents the requirement for a functional *csrA* allele raised the possibility that *tnaA* might act downstream of CsrA in a putative regulatory pathway. To test this possibility, we assayed tryptophanase activity by measuring the hydrolysis of the chromogenic tryptophan analogue *S*-*O*-nitrophenyl-L-cysteine (SOPC) to *O*-nitrothiophenolate (ONTP) in bacterial lysates that had been precultivated on agar plates containing or lacking tryptophan, essentially as described previously (2). Accordingly, tryptophanase activity was dramatically reduced in the *csrA* mutant (Fig. 1E). Moreover, this effect occurred independently of the addition of exogenous tryptophan (Fig. 1E). Collectively, these results suggest that the inability of the *csrA* mutant to paralyze and kill the nematode results from reduced tryptophanase activity and that *tnaA* acts distally to *csrA*.

In *E. coli*, the *tnaCAB* operon is subject to transcriptional as well as posttranscriptional control (9, 11, 12, 16, 36, 81). The nascent leader peptide, TnaC, while translocating through the exit tunnel of the ribosome, transduces conformational alterations in the ribosome to generate a stereospecific L-tryptophan-binding site near the peptidyltransferase center (79). Bound tryptophan promotes ribosomal stalling, which in turn masks the *boxA*-*rut* riboelement of the transcriptional terminator Rho that overlaps the C terminus as well as the segment immediately downstream of the *tnaC* open reading frame (ORF) (35, 37, 80). Consequently, Rho does not bind to the transcript and the stalled RNA polymerase is not offloaded and continues to transcribe the downstream genes *tnaA* and *tnaB* (35–38). Thus, tryptophan posttranscriptionally induces the expression from the *tnaCAB* operon in *E. coli* (79). The primary structure of the TnaC leader peptide as well as the nucleotide sequence of the *boxA*-*rut* site within the *tnaCAB* operon of EPEC and EHEC are identical to that of *E. coli* K-12, suggesting that tryptophan-mediated stimulation of the *tna* operon is likely conserved (Fig. 2A). Consistent with this bioinformatic observation, a modest but reproducible increase in tryptophanase activity was observed upon addition of tryptophan to LB medium (Fig. 1E and 2D). LB medium is naturally replete with tryptophan in the form of tryptone, and thus its presence likely masks the actual induction in tryptophanase activity by exogenously added tryptophan.

Because uptake of tryptophan is necessary for killing of *C. elegans*, we reasoned that tryptophan importers might also be necessary for toxin production. In *E. coli*, three permeases, *tnaB*, *aroP*, and *mtr*, are responsible for importing tryptophan into the bacterium (94). Orthologs of all the three transporters are present in EPEC (data not shown). Using lambda red-mediated recombineering, we substituted *mtr* and *aroP* with a *cat* cassette as described previously (7, 22, 63) and evaluated the roles of each of the permeases in toxin production and pathogenesis in *C. elegans*. Inactivation of *mtr* or *aroP* did not compromise the ability of EPEC to paralyze or kill *C. elegans*

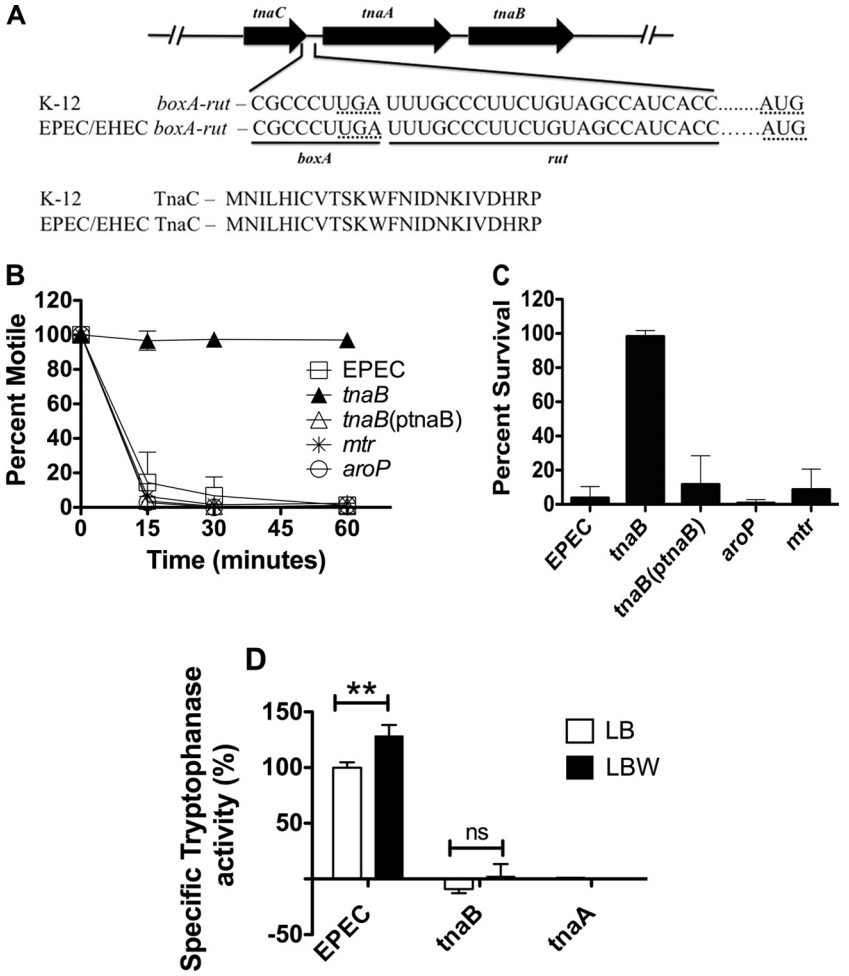

FIG. 2. (A to D) TnaB but not AroP or Mtr imports tryptophan into EPEC to stimulate toxin-dependent killing of *C. elegans*. (A) Comparative analysis of the primary structure of the *boxA-rut* riboelement and the TnaC leader peptide of the *tnaCAB* operon in *E. coli* K-12 and EPEC/EHEC. The translation termination codon of *tnaC* and the translation initiation codon of *tnaA* are indicated by dashed underlines. The intersite distance between the terminal nucleotide of *rut* and the translational initiation nucleotide of *tnaA* is 197 bases. (B and C) Young adult worms were exposed to EPEC and its congenic mutant derivatives, the *tnaB*, *mtr*, and *aroP* mutants and the *tnaB* complemented strain [*tnaB*(ptnaB)], and assayed for paralysis (B) and killing (C). Error bars indicate the standard deviations of results from at least three independent experiments, with each using at least two replicates. A one-way ANOVA was used to assess statistical significance. A P value cutoff of <0.05 was considered statistically significant. The calculated P values for both the paralysis and killing assays were <0.02. (D) Specific tryptophanase activity was assayed in the *tnaB* mutants as described above. The unpaired Student t test was employed to assay for statistical significance between the indicated samples. A P value cutoff of <0.01 was considered statistically significant. **, $P < 0.01$; ns, no statistically significant difference.

(Fig. 2B and C). In contrast, inactivation of *tnaB* was sufficient to completely abolish EPEC-induced paralysis and killing of *C. elegans* (Fig. 2B and C). The *tnaB* mutant regained its pathogenicity when complemented with a functional *tnaB* allele that was expressed under a heterologous promoter from the low-copy-number plasmid ptnaB (Fig. 2B and C) (Tables 1 and 2). The attenuated phenotype of the *tnaB* mutant correlated with reduced tryptophanase activity (Fig. 2D). Moreover, the tryp-

TABLE 2. Oligonucleotides used in this study

Primer	Sequence[b]
5′-aroP-P2-Wanner-EPEC	CCGCCACATACAGCTTATCGCGCTGGGAGGCGCGATAGGGACAGGCATATGAATATCCTCCTTA
3′-aroP-P1-Wanner-EPEC	TACCTAACACGATCAGCCATACCGGGATCAGGTATACCGAAATCGGTGTAGGCTGGAGCTGCTTC
5′-mtr-P2-Wanner-EPEC	TTATCGGCGGCACCATTATTGGCGCAGGGATGTTTTCTCTGCCAGCATATGAATATCCTCCTTA
3′-mtr-P1-Wanner-EPEC	CATTGTGTAGGCAGCAGAAATGTCGGATAAGGCACCGCTGATTACGTGTAGGCTGGAGCTGCTTC
5′-tnaB-EcoRI-pBAD24	gcggccGAATTCCCTCTAAAGGTGGCATCATGACTG
3′-tnaB-PstI-pBAD24	gcggccCTGCAGAAAGCGGGACATGGGCTAAAG
c1[a]	TTATACGCAAGGCGACAAGG
c2[a]	GATCTTCCGTCACAGGTAGG

[a] See reference 22.
[b] Underlined sequences indicate restriction sites. Lowercase indicates additional nucleotides that facilitate cleavage of the PCR product by the restriction enzyme.

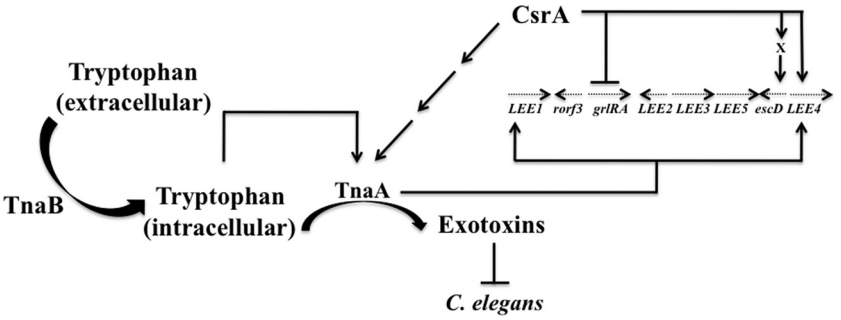

FIG. 3. Model for the role of CsrA and TnaB in the regulation of *tnaA* and toxin production. CsrA positively regulates tryptophanase activity independently of the tryptophan permease TnaB. TnaB is the primary importer of tryptophan when EPEC is cultivated on LB medium. Imported tryptophan stimulates the expression of *tnaA*, as is evident by elevated tryptophanase activity. In turn, tryptophanase catabolizes tryptophan to synthesize exotoxins, which paralyze and kill *C. elegans. csrA* and *tnaA* also regulate the *LEE* in A/E pathogens. Activating and repressive circuits are depicted as thin lines with arrowheads and blunt ends, respectively. Arrows with dashed lines represent the LEE. Curved arrows indicate catalytic reactions.

tophan-mediated induction of *tnaA* was no longer evident when *tnaB* was inactivated (Fig. 2D). Taken together, these results suggest that on LB agar, TnaB is the primary permease responsible for importing tryptophan into the bacterium, which subsequently induces *tnaA*. Besides inducing *tnaA*, tryptophan is also one of the natural substrates of tryptophanase (65, 66). Because overexpression of *tnaA* in LB medium, without added tryptophan, is insufficient for worm killing, tryptophan must play an important role as a tryptophanase substrate and as a precursor for exotoxin synthesis. Interestingly, inactivation of *csrA* does not disrupt the tryptophan-mediated stimulation of tryptophanase (Fig. 1E), suggesting that the import of the inducer remains unhindered in the *csrA* mutant. This corroborates the observation that overexpression of *tnaA*, without *tnaB*, is sufficient to restore virulence to the *csrA* mutant when cultivated on LB agar supplemented with tryptophan (LBW) (Fig. 1C and D). Curiously, tryptophan repressed tryptophanase activity when *csrA* was overexpressed (Fig. 1E). Biochemical studies with tryptophanase from *E. coli* suggest that the degradative product of tryptophan, indole, exerts a dose-dependent, feedback inhibitory effect on the enzymatic activity by competing with its substrates for the catalytic site (39). Moreover, derivatives of indole have also been demonstrated to silence the expression of *tnaA* (53). Thus, the observed phenotype likely stems from the repressive effect of elevated indole levels on the expression and/or activity of tryptophanase. In summary, our results suggest that CsrA and TnaB exert their effects via parallel pathways that converge at the level of regulation of *tnaA* to synthesize exotoxins that enable EPEC to paralyze and kill *C. elegans* (Fig. 3).

Herein, we provide evidence that the dual metabolic and virulence regulator CsrA, previously shown to regulate the virulence of EPEC in mammals (7), also contributes to pathogenicity in nematodes. Our results also suggest that toxin-based bioassays employing *C. elegans* can be effectively utilized to identify novel virulence factors of A/E pathogens with relevance to mammalian pathogenesis. Future experiments utilizing a saturated transposon-mutagenized library will provide invaluable insight into evolutionarily conserved virulence determinants of EPEC. Moreover, using worm killing as a readout, we were able to determine the metabolic requirement of the different tryptophan importers in the nematocidal activity of EPEC. Thus, it may be possible to adapt the toxin-based assay to study alternative metabolic pathways and design screens to identify virulence factors for other pathogens. For instance, the murine A/E pathogen *C. rodentium* lacks *tnaA*. However, the closely related enzyme tyrosine phenol lyase (*tpl*) is present in the genus *Citrobacter* (29, 43). Both the enzymes utilize the same cofactors and display remarkable conservation of key residues (6). Tpl enzymatically cleaves tyrosine to yield phenol, pyruvate, and ammonia. Because phenolic compounds are nematotoxic (50, 87), substitution of tryptophan with tyrosine in the medium may facilitate evaluation of the toxicity of *C. rodentium* toward *C. elegans* and identifying virulence factors that may also induce pathology in mammals.

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