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MOLECULAR PATHOGENESIS

Vibrio cholerae TolC Is Required for Expression of the ToxR Regulon

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ABSTRACT *Vibrio cholerae* is a Gram-negative bacterium that causes the enteric disease cholera. *V. cholerae* colonization of the human intestine is dependent on the expression of both virulence genes and environmental adaptation genes involved in antimicrobial resistance. The expression of virulence genes, including the genes encoding the main virulence factors cholera toxin (CT) and the toxin-coregulated pilus (TCP), are coordinately regulated by the ToxR regulon. Tripartite transport systems belonging to the ATP binding cassette, major facilitator, and resistance-nodulation-division families are critical for *V. cholerae* pathogenesis. Transport systems belonging to these families contribute to myriad phenotypes, including protein secretion, antimicrobial resistance, and virulence. TolC plays a central role in bacterial physiology by functioning as the outer membrane pore protein for tripartite transport systems. Consistent with this, *V. cholerae tolC* was previously found to be required for MARTX toxin secretion and antimicrobial resistance. Here, we investigated the contribution of TolC to *V. cholerae* virulence. We documented that *tolC* was required for CT and TCP production in O1 El Tor *V. cholerae*. This phenotype was linked to repression of the critical ToxR regulon transcription factor *aphA*. Decreased *aphA* transcription correlated with increased expression of the LysR-family transcription factor *leuO*. Deletion of *leuO* restored *aphA* expression, and CT and TCP production, in a *tolC* mutant. The collective results document that *tolC* is required for ToxR regulon expression and further suggest that *tolC* participates in an efflux-dependent feedback circuit to regulate virulence gene expression.

KEYWORDS TolC, cholera, virulence, RND, efflux, TolC, *Vibrio cholerae*

Vibrio cholerae is a Gram-negative human pathogen that causes the acute diarrheal disease cholera (1). Cholera affects millions of people each year, particularly people in areas with poor sanitation. *V. cholerae* is a common inhabitant of fresh and saltwater environments from which people acquire cholera through the ingestion of *V. cholerae* contaminated water or food (2–4). Following ingestion, *V. cholerae* activates the production of virulence factors in the small intestine that facilitate colonization and the development of a severe secretory diarrhea that is the hallmark of the disease cholera.

The production of virulence factors by *V. cholerae* is under the transcriptional control of a hierarchical regulatory system called the ToxR regulon (5). The masthead of this regulon is ToxR, which was the first gene identified in the regulon (6). ToxR is a membrane-associated transcriptional regulator that modulates virulence factor production in response to environmental cues in the host. ToxR functions in conjunction with ToxS, which is encoded downstream from *toxR* and functions to stabilize ToxR (7–9). ToxR contains three domains: a periplasmic sensing domain (PPD) that is connected to a cytoplasmic DNA-binding domain by a single transmembrane-spanning domain (7, 10–12). Environmental sensing by ToxR is thought to be mediated through its PPD, which interacts with chemical cues in the periplasm to affect the activity of the

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DNA-binding domain at target genes. In the presence of activating signals, ToxR binds to the *toxT* promoter with another membrane-associated transcriptional regulator, TcpP, resulting in activation of *toxT* transcription. Expression of *tcpP* is regulated by two environmentally responsive transcription factors, AphA and AphB (13, 14). Expression of *aphA* is regulated by quorum sensing (13), while *aphB* is transcriptionally regulated by OmpR and posttranscriptionally by oxygen (15, 16). Once ToxT is produced, it directly activates the expression of the genes responsible to produce the two primary *V. cholerae* virulence factors: the toxin coregulated pilus (TCP) and cholera toxin (CT). TCP is a type IV pilus that is essential for *V. cholerae* colonization of the small intestine, whereas CT is an enterotoxin that is responsible for production of secretory diarrhea.

TolC is an outer membrane protein that was first identified in *Escherichia coli* (17). TolC is the archetype of a large family of outer membrane proteins belonging to the outer membrane efflux protein (OEP) superfamily that are ubiquitous among Gram-negative bacteria (18, 19). TolC plays critical roles in cellular physiology by serving as the outer membrane pore protein for transport systems belonging to the ATP-binding cassette (ABC), major facilitator (MF), and resistance-nodulation-division (RND) families. Transporters belonging to these families contribute to a multitude of phenotypes in Gram-negative bacteria, including antimicrobial resistance, metal homeostasis, protein secretion, metabolism, and virulence (20). This is true in *V. cholerae*, where deletion of *tolC* prevented MARTX toxin secretion and rendered the mutant strain hypersensitive to multiple antibiotics and highly attenuated for colonization of the infant mouse small intestine (21). These observations suggested that *V. cholerae tolC* was essential for both virulence and antimicrobial resistance, but the mechanism by which *tolC* contributed to virulence was not explored.

In this study, we investigated the contribution of *V. cholerae* TolC in virulence factor production in O1 El Tor strain N16961. We found that *tolC* was required for robust expression of the *V. cholerae* ToxR virulence regulon. Mutation of *tolC* resulted in attenuated CT and TCP production. This defect was linked to the LeuO-dependent repression of *aphA*. The collective results revealed that TolC has pleiotropic contributions to *V. cholerae* virulence, being required for virulence gene expression, MARTX toxin secretion, and resistance to antimicrobial compounds that are present in the host.

RESULTS

***V. cholerae tolC* is required for virulence factor production.** Previous studies showed that a *V. cholerae tolC* mutant was highly attenuated for colonization of the infant mouse small intestine (21). This phenotype was attributed to increased antimicrobial sensitivity, but the contribution of *tolC* to virulence factor production was not assessed. Therefore, we tested the effect of *tolC* deletion on CT and TCP production in *V. cholerae* O1 El Tor strain N16961. We cultured N16961 and an isogenic Δ*tolC* mutant under virulence-inducing conditions (i.e., AKI conditions) and quantified CT and TcpA production as described in Materials and Methods (22). TcpA is the pilin subunit of the TCP and is used as a marker for TCP production (23). The results showed that *tolC* deletion resulted in an ~66% reduction in CT production relative to the wild type (WT), with a corresponding decrease in TcpA production in the *tolC* mutant (Fig. 1A). Complementation of the Δ*tolC* mutant with pBAD18-*tolC* revealed that ectopic expression of *tolC* partially complemented the Δ*tolC* mutant for both CT and TcpA production (Fig. 1B). It is unknown why we only observed partial complementation, but we suspect that it may be due to overexpression toxicity, as ectopic overexpression of *tolC* was detrimental to cell growth. From these results we concluded that *tolC* is required for high-level production of virulence factors in *V. cholerae*.

TolC is required for induction of the ToxR regulon. Deletion of *tolC* resulted in diminished CT and TcpA production. As CT and TCP are under the control of the ToxR regulon, this finding suggested that the mutation of *tolC* impacted the expression of the ToxR regulon. To test this, we introduced luciferase-based transcriptional reporters for CT and TCP production (i.e., *ctxA* and *tcpA*) and the five major ToxR regulon

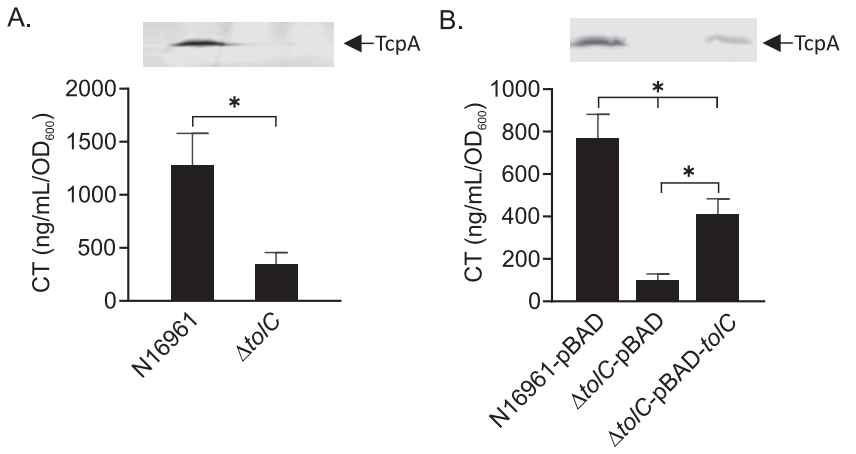


FIG 1 TolC is required for CT and TCP production in *V. cholerae*. The indicated *V. cholerae* strains were cultured under AKI conditions for 18 h, when culture aliquots were then collected and assayed for CT and TCP production (inset) as described in Materials and Methods. (A) CT and TcpA production in WT and Δ*tolC* strains. (B) Complementation of CT and TcpA production (inset) in the Δ*tolC* mutant by ectopic *tolC* expression from the arabinose-regulated promoter in pBAD18. The CT results are the mean and standard deviation from three independent experiments. Statistical significance was determined using Student's *t* test (A) and by analysis of variance (ANOVA) with the Tukey-Kramer multiple-comparison test (B). The TcpA Western blot is representative of three independent experiments. *, $P \leq 0.05$.

regulatory genes (i.e., *aphA*, *aphB*, *toxT*, *tcpP* and *toxR*) into N16961 and an isogenic Δ*tolC* mutant (Table 1). The resulting strains were then cultured under virulence-inducing conditions for 5 h, when gene expression was quantified. Five hours was selected as the time point to measure gene expression to capture effects on both early- and late-expressed genes in the hierarchical ToxR regulon. The results showed that *ctxA* and *tcpA* expression were reduced in the *tolC*-negative background relative to the WT (Fig. 2). This is consistent with the CT enzyme-linked immunosorbent assay (ELISA) and TcpA Western blot results presented in Fig. 1 and suggests that attenuated CT and TcpA production in the Δ*tolC* mutant results from decreased transcription and not from posttranscriptional regulation or a defect in protein secretion.

We next assessed the expression of the genes that are upstream of *ctxA* and *tcpA* in the ToxR regulon. This revealed that expression of *toxT*, the direct regulator of *ctxA* and *tcpA*, was reduced in the *tolC* mutant (Fig. 2). Expression of *toxT* is under the control of ToxR and TcpP. While *toxR* transcription was unchanged in the *tolC* mutant (Fig. 2), the expression of *tcpP* was significantly reduced in the Δ*tolC* strain compared to the WT. The production of TcpP is regulated by AphA and AphB, which function synergistically at the *tcpP* promoter to activate its expression. The results here showed that the expression level of *aphA*, but not *aphB*, was reduced in the Δ*tolC* mutant (Fig. 2). These findings suggest that the attenuated CT and TCP production in the Δ*tolC* mutant results from decreased *aphA* transcription.

Deletion of *tolC* activates *leuO* transcription to repress virulence. The expression of *aphA* is negatively regulated by quorum sensing and the LysR family transcriptional regulator LeuO (13, 24). However, the fact that N16961 is quorum sensing negative (25) strongly suggested that quorum sensing was not involved in virulence repression in the Δ*tolC* mutant. Therefore, we examined the effect of *tolC* deletion on *leuO* expression by introducing a *leuO-lux* transcriptional reporter (pTB32) into WT and Δ*tolC* strains. The resulting strains were cultured under virulence-inducing conditions for 5 h, when *leuO* expression was quantified. The results revealed a >2-fold increase in *leuO* expression in the Δ*tolC* mutant relative to the WT (Fig. 3A). As LeuO directly binds the *aphA* promoter to repress its transcription, these results suggest that LeuO was responsible for *aphA* repression and attenuated virulence factor production in the Δ*tolC*

TABLE 1 Strains, plasmids, and oligonucleotide primers

Strain, plasmid, or primer	Description	Reference or source
E. coli		
EC100	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80d*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara*, *leu*)*7697 galU galK* λ^- *rpsL* (Sm^r) *nupG pir*$^+$	Epicentre
SM10 *λpir*	*thi-1 thr leu tonA lacY supE recA*::RP4-2-4-Tc::Mu Km^r (*λ pir*R6K)	Lab collection
V. cholerae		
JB58	01 El Tor strain N16961, Δ*lacZ* Sm^r	25
JB150	JB58 Δ*tolC*	21
JB485 (ΔRND)	JB58 Δ*vexB* Δ*vexD* Δ*vexF* Δ*vexH* Δ*vexK* Δ*vexM*	34
YW535	JB58 Δ*tolC* Δ*leuO*	This study
YW576	JB485 ΔtolC	This study
C6706	01 El Tor strain C6706, Sm^r	Lab collection
Plasmids		
pBAD18	Arabinose-inducible expression vector, Amp^r	61
pBAD18-*tolC*	pBAD18 expressing *tolC* from *V. cholerae* strain N16961, Amp^r	21
pWM91-Δ*tolC*	*oriR6k* allelic exchange vector used to construct in-frame deletion of *tolC*, *mob-oriT sacB* Amp^r	21
pWM91-Δ*leuO*	*ori*R6k allelic exchange vector used to construct in-frame deletion of *leuO*, *mob-oriT sacB* Amp^r	24
pBBR-*lux*	*luxCDABE*-based promoter fusion vector, Cm^r	62
TB085	JB58-pTB17 (*toxT-lux*), Amp^r	52
TB091	JB58-pTB20 (*tcpP-lux*), Amp^r	52
TB092	JB58-pTB22 (*tcpA-lux*), Amp^r	52
TB093	JB58-pTB23 (*ctxA-lux*), Amp^r	52
TB096	JB150-pTB17 (*toxT-lux*), Amp^r	This study
TB097	JB150-pTB22 (*tcpA-lux*), Amp^r	This study
TB098	JB150-pTB23 (*ctxA-lux*), Amp^r	This study
TB099	JB150-pTB19 (*aphA-lux*), Amp^r	This study
TB100	JB150-pTB20 (*tcpP-lux*), Amp^r	This study
TB102	JB58-pTB19 (*aphA-lux*), Amp^r	52
TB103	JB150-pTB21 (*toxR-lux*), Amp^r	This study
TB104	JB58-pTB21 (*toxR-lux*), Amp^r	52
TB106	JB58-pTB18 *lux*-based promoter probe vector, Amp^r	52
TB107	JB150-pTB18 *lux*-based promoter probe vector, Amp^r	This study
TB126	JB58-pTB25 (*aphB-lux*), Amp^r	52
TB127	JB150-pTB25 (*aphB-lux*), Amp^r	This study
TB139	JB150-pTB32 (*leuO-lux*), Amp^r	This study
TB140	JB58-pTB32 (*leuO-lux*), Amp^r	52
YV551	YW535-pTB22 (*tcpA-lux*), Amp^r	This study
YV553	YW535-pTB17 (*toxT-lux*), Amp^r	This study
YV585	YV576-pTB19 (*aphA-lux*), Amp^r	This study
YV587	YV576-pTB23 (*ctxA-lux*), Amp^r	This study
Oligonucleotide primers		
AmpR-F	GCCCGCCTGATGAATGCTCATCCGGGAATTCTGACGGATGGCCTTTTTGCGTTTCT	
AmpR-R	CTCACCGTCTTTCATTGCCATACGGGAATTCTACAGGGCGCGTAAATCAATCTAAAG	
61T-F-SacI	ATGAGCTCGTTTGACAGCTTATCATCGGAGCTC	
61T-R-BamHI	TTGGATCCGTCGGGATCGCTAGTTAGTTAGG	
VC0164-qRT-F	CTGCTGCGCGAACGTAGTAG	
VC0164-qRT-R	ACGTAAATGGCGTCTCAACCC	
VC0629-qRT-F	GCTGCCGATTAAAGTCGAAGG	
VC0629-qRT-R	ACAATGGCGGGTTTACCATCTA	
VC0914-qRT-F	TTGCTCGATCGGTTTAGCTTGA	
VC0914-qRT-R	GTTCTGGCTTGATGCGTACATAC	
VC1673-qRT-F	CTTTAGCATCACCGGATGACTCAT	
VC1673-qRT-R	AACCGCATCCACGTTACAAC	
VC1757-qRT-F	GCTGATGCGCTATAACGGTCAG	
VC1757-qRT-R	CGCCGGTGACATTCGAGATA	
VCA0638-qRT-F	TGCCGTACAGTGGGCTATCC	
VCA0638-qRT-R	ACCCGCATGGATGATTACATCG	

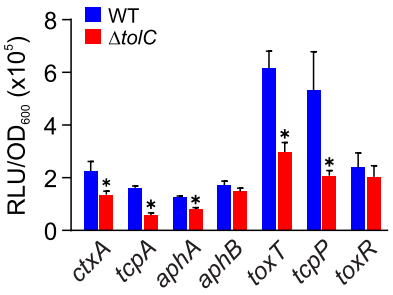


FIG 2 Effect of *tolC* on the expression of genes in the ToxR virulence regulon. *V. cholerae* N16961 (blue bars) and an isogenic Δ*tolC* mutant (blue bars) bearing the indicated ToxR regulon luciferase-based reporters were cultured under AKI conditions for 5 h when gene expression was quantified as relative light units (RLU) divided by the optical density at 600 nm. The results represent the average of RLU/OD and standard deviation from three independent experiments. Statistical significance was determined using Student's *t* test. *, $P \leq 0.05$ relative to the WT.

mutant. If this was true, we hypothesized that deletion of *leuO* in the Δ*tolC* background should restore the expression of *aphA*, *ctxA*, and *tcpA* in the Δ*tolC* mutant background. We therefore generated a Δ*tolC* Δ*leuO* double mutant and cultured WT, Δ*tolC*, and Δ*tolC* Δ*leuO* mutants bearing transcriptional reporters for *aphA*, *ctxA*, and *tcpA* under virulence-inducing conditions for 5 h before quantifying reporter expression. The results showed that *aphA*, *ctxA*, and *tcpA* expression was attenuated in the Δ*tolC* mutant and that deletion of *leuO* in the Δ*tolC* background restored WT-level expression of all three genes (Fig. 3B). The expression of *aphA* increased in the Δ*tolC* Δ*leuO* mutant relative to the WT, which is consistent with previous studies showing that LeuO directly bound to the *aphA* promoter to repress its expression (12). Consistent with the reporter data, deletion of *leuO* in the Δ*tolC* background restored both CT and TcpA production to WT levels (Fig. 3C). Taken together, these results supported the conclusion

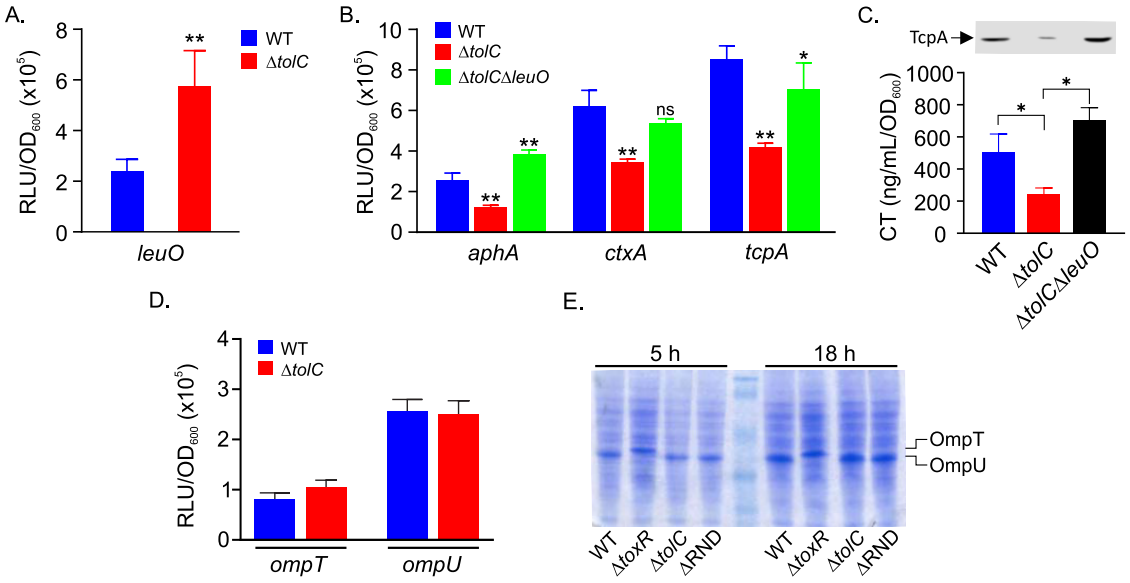
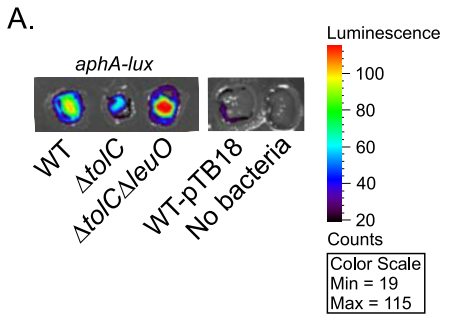


FIG 3 TolC-dependent virulence repression is mediated by LeuO. *V. cholerae* N16961 strain JB58 and isogenic Δ*tolC* and Δ*tolC* Δ*leuO* mutants bearing *lux*-based transcriptional reporters for *leuO*, *aphA*, *ctxA*, *tcpA*, *ompT*, and *ompU* were cultured under AKI conditions for 5 h when gene expression was quantified as relative light units (RLU) divided by the optical density at 600 nm. (A) Effect of *tolC* mutation on *leuO* expression in WT and Δ*tolC* strains. (B) Expression of *aphA*, *ctxA*, and *tcpA* in WT, Δ*tolC*, and Δ*tolC* Δ*leuO* strains. (C) CT and TcpA production (inset) in the indicated strains following overnight growth under AKI conditions. (D) Effect of *tolC* deletion on *ompT* and *ompU* expression. (E) SDS-PAGE gel stained with Coomassie blue. Whole-cell lysates of the indicated *V. cholerae* strains were prepared following growth under AKI conditions for 5 h or 18 h and resolved by SDS-PAGE. The OmpT and OmpU protein bands are indicated on the right. The results represent the average RLU/OD and standard deviation from three independent experiments. Statistical significance was determined using Student's *t* test (A) and a one-way ANOVA with Dunnett's *post hoc* test (B and C). *, $P < 0.01$ relative to WT. **, $P < 0.001$; ns, not significant.



B.

Inoculum	RLU
WT	4.37×10^5
Δ*tolC*	1.63×10^5
Δ*tolC*Δ*leuO*	4.75×10^5
WT-pTB18	1.31×10^5
No bacteria	2.48×10^4

FIG 4 TolC affects *aphA* expression in an *ex vivo* small-intestine colonization model. The indicated *V. cholerae* N16961 strains bearing pTB18 (empty vector) or pTB19 (*aphA-lux*) were inoculated onto the surface of fresh porcine small intestine as described in Materials and Methods. The inoculated intestine segments were then incubated at 37°C in the absence of oxygen. After 5 h, the intestinal segments were imaged for luminescence production on an IVIS imaging system (A) with luminescence production being quantified using the Living Image software (B).

that *tolC* deletion resulted in increased *leuO* expression and that *leuO* was responsible for virulence attenuation in the Δ*tolC* mutant.

ToxR reciprocally regulates the expression of the *ompT* and *ompU* porins independently of TcpP (26–28), with ToxR activating *ompU* and repressing *ompT* during growth in rich medium (29, 30). ToxR-dependent induction of *ompU* was reported to be activated in response to bile salts and certain amino acids, with the latter resulting from increased *toxRS* expression (31–33). As our data suggested that *toxRS* transcription was not affected in the Δ*tolC* mutant, we tested whether *tolC* deletion affected the expression of porin transcription or production. We first introduced *ompT* and *ompU* transcriptional reporters into WT and Δ*tolC* strains. We then cultured the resulting strains under AKI conditions for 5 h before quantifying gene expression. The results showed that there was no significant difference in *ompU* or *ompT* expression in the Δ*tolC* mutant relative to the WT (Fig. 3D). We next examined OmpT and OmpU production using SDS-PAGE of whole-cell lysates from 5-h and 18-h cultures of the Δ*tolC* and ΔRND mutants. As ToxR activates *ompU* transcription and represses *ompT* transcription in rich media, we included the WT and the Δ*toxRS* mutant to discriminate between the OmpT and OmpU protein bands on the SDS-PAGE gel. The results showed that OmpU was the major protein in WT lysates, whereas OmpT was the major protein in the Δ*toxRS* mutant at both time points, which is consistent with previous reports (Fig. 3E). Porin production in the Δ*tolC* and ΔRND mutants was indistinguishable from the WT at both time points. These results indicated that neither *tolC* mutation nor impaired RND-mediated efflux affected ToxR-dependent porin regulation under the tested conditions. Thus, it appears that the *tolC* mutant phenocopies the RND efflux null mutant in activating *leuO* transcription without affecting porin expression.

TolC is required for robust *aphA* expression in an *ex vivo* intestine colonization model. We sought to assess whether *tolC* affected *aphA* expression *in vivo*, but the *tolC* mutant exhibits a >500-fold increase in susceptibility to bile acids and other detergents relative to the WT, which precludes its ability to colonize infant mice (21, 34). We therefore tested the effect of *tolC* on *aphA* expression using an *ex vivo* small-intestine colonization model as previously described (35). To accomplish this, WT *V. cholerae* and isogenic Δ*tolC* and Δ*tolC* Δ*leuO* mutants bearing empty vector (pTB18) or *aphA-lux* (pTB19) were spotted onto the luminal surface of ~1.0-cm-square segments of fresh porcine small intestine. The inoculated intestinal segments were then overlaid with LB broth and incubated at 37°C under anaerobic conditions. After a 5-h incubation, *aphA* expression on the surface of the intestinal segments was captured as emanating luminescence using an IVIS Lumina X5 imaging system (Fig. 4A), and luminescence production was quantified using the Living Image software (Fig. 4B). The results revealed the induction of *aphA* in the intestine segments inoculated with WT *V. cholerae* (Fig. 4). The level of *aphA* expression decreased by approximately 2.7-fold in the

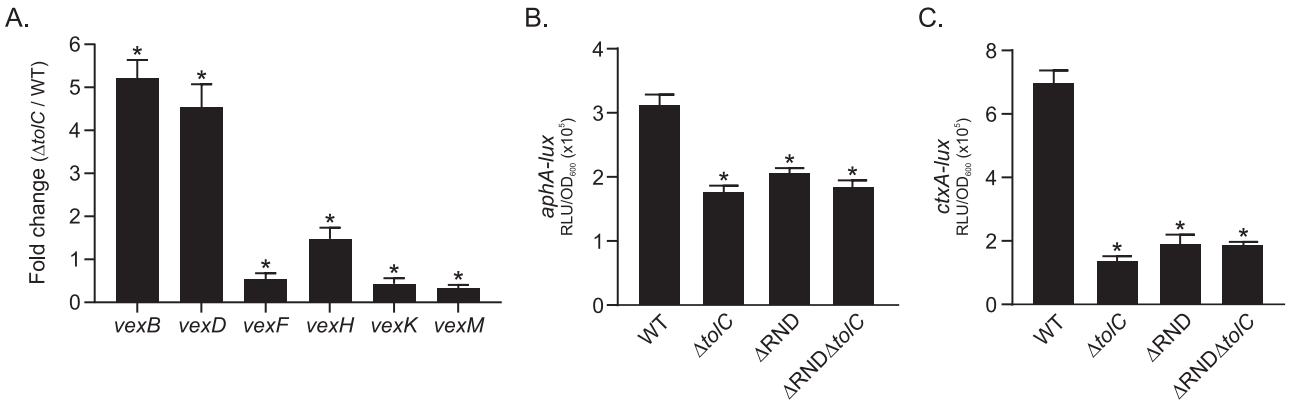


FIG 5 Effect of *tolC* on the expression of the *V. cholerae* RND efflux systems. (A) The expression level of the genes encoding the six *V. cholerae* RND-family pump proteins in the Δ*tolC* mutant relative to the WT. *V. cholerae* WT and Δ*tolC* strains were cultured under AKI conditions for 5 h, and total RNA was isolated and used to assess the expression level of the indicated genes by qRT-PCR as described in Materials and Methods. The results are the means and standard deviations from three independent experiments. Statistical significance was determined by the *t* test relative to a hypothetical fold change of 1.0. *, $P \leq 0.05$. (B and C) Expression of *aphA* and *ctxA* in WT, Δ*tolC*, ΔRND, and ΔRND Δ*tolC* strains following growth under AKI conditions for 5 h. The indicated strains bearing *aphA-lux* (B) or *ctxA-lux* (C) reporters were cultured under AKI conditions for 5 h, when reporter gene expression was quantified as relative light units (RLU) and normalized by the optical density at 600 nm. The results are the means and standard deviations from three independent experiments. Statistical significance was determined using ANOVA with the Tukey-Kramer multiple-comparison test. *, $P \leq 0.05$ relative to the WT.

Δ*tolC* mutant relative to the WT, while deletion of *leuO* in the Δ*tolC* background (i.e., Δ*tolC* Δ*leuO* mutant) restored *aphA* expression to a level that was slightly greater than that of the WT (Fig. 4). There was low luminescence production in the empty vector control (i.e., WT-pTB18) and uninoculated control (Fig. 4). As there is no difference in the growth kinetics between these strains *in vitro* (not shown), these results suggest that *tolC* is required for robust *aphA* expression during *V. cholerae* colonization of the intestinal epithelium. These results are also consistent with the reporter studies described above (Fig. 3) and suggest that this *tolC*-dependent regulatory circuit is relevant *in vivo*.

Deletion of *tolC* affects the expression of RND-family transporters. The RND efflux systems are ubiquitous tripartite transporters in Gram-negative bacteria that contribute to a multitude of phenotypes, including antimicrobial resistance and virulence (36). In *V. cholerae*, TolC is believed to function as the outer membrane pore protein for all six of the *V. cholerae* RND efflux systems (18, 21). As the RND efflux systems are regulated by a feedback mechanism in response to their efflux substrates and *tolC* is required for their function, we tested if mutation of *tolC* affected the expression of the *V. cholerae* RND efflux systems. We therefore cultured WT and Δ*tolC* strains under AKI conditions for 5 h, when cell pellets were used to isolate total RNA. We then used quantitative reverse transcription-PCR (qRT-PCR) to assess the expression of each of the six *V. cholerae* RND efflux pump genes (i.e., *vexB*, *vexD*, *vexF*, *vexH*, *vexK*, and *vexM*). The results showed that the expression of *vexB*, *vexD*, and *vexH* increased in the Δ*tolC* mutant relative to the WT, with *vexB* and *vexD* being upregulated 5.2- and 4.7-fold (Fig. 5A), respectively. The increased expression of *vexH* was more moderate (~1.5-fold). In contrast, the expression level of the *vexF*, *vexK*, and *vexM* efflux pump genes decreased by ~2-fold relative to the WT. A similar feedback response among the *vexB*, *vexD*, and *vexH* RND efflux systems was noted in transcriptomics experiments comparing an RND-negative *V. cholerae* strain to the WT (12), which is consistent with the RND systems being regulated by a feedback mechanism. Taken together, these results provide additional support for the conclusion that *tolC* serves as the outer membrane pore for the *V. cholerae* RND efflux systems (21, 34).

To further confirm that the Δ*tolC* strain phenocopied the RND efflux-deficient mutant, we deleted *tolC* in RND efflux pump-deficient strain JB485 (i.e., ΔRND) and then quantified *aphA* and *ctxA* expression in WT, Δ*tolC*, ΔRND, and ΔRND Δ*tolC* strains following growth

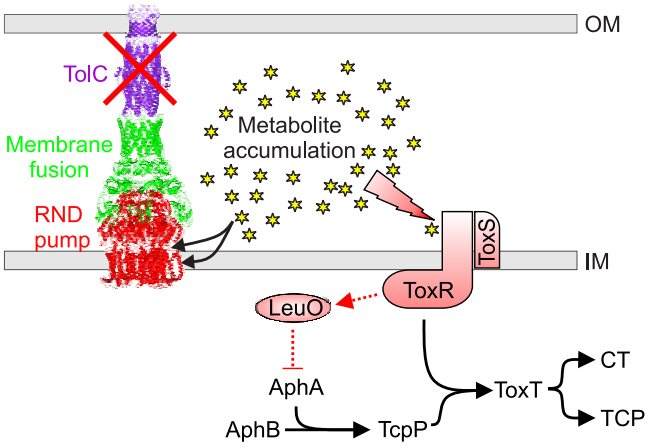


FIG 6 Working model for the function of TolC in *V. cholerae* virulence factor production. TolC functions as the outer membrane pore protein for the *V. cholerae* RND family efflux systems. In the absence of TolC, the RND family transporters no longer function and metabolites that are normally removed from the cell by the RND transporters accumulate intracellularly. The accumulated metabolites then interact with periplasmic sensors, including ToxR, to activate *leuO* transcription. LeuO is a repressor that binds to the *aphA* promoter to downregulate the ToxR regulon, leading to attenuated CT and TCP production. The RND efflux system image depicts the *E. coli* AcrAB-TolC system and is derived from PDB entry 5V5S (60).

under virulence-inducing conditions. Like the *leuO* and porin results described above, the data showed that the Δ*tolC* mutant phenocopied ΔRND for both *aphA* and *ctxA* expression. Furthermore, deletion of *tolC* in ΔRND (i.e., the ΔRND Δ*tolC* mutant) did not impact the expression of either *aphA* or *ctxA* relative to that of the ΔRND or Δ*tolC* mutants (Fig. 5B and C). The fact that the ΔRND Δ*tolC* mutant phenocopied the ΔRND or Δ*tolC* mutant suggests that these mutants impact virulence via the same mechanism. From these results we concluded that virulence attenuation in the Δ*tolC* strain very likely resulted from the functional inactivation of RND-mediated efflux due to the loss of the RND system's requisite outer membrane pore protein (i.e., TolC).

DISCUSSION

Here, we investigated the function of TolC in *V. cholerae* virulence factor production. We documented that deletion of *tolC* in O1 El Tor strain N16961 resulted in attenuated production of the critical virulence factors CT and TCP. Decreased CT and TCP production correlated with the activation of a negative regulatory circuit that resulted in increased expression of the LysR-family transcription factor *leuO*. LeuO was linked to repression of *aphA* transcription, which led to downregulation of the genes that are downstream of *aphA* in the ToxR regulon (Fig. 6). Confirmation of this regulatory circuit was provided by complementation studies and results showing that deletion of *leuO* in the *tolC* null background restored both *aphA* expression and virulence factor production. Collectively these results expand the function of TolC in *V. cholerae* biology and suggest that TolC has pleiotropic contributions to virulence.

TolC functions as the outer membrane pore for multiple classes of transporters, including ABC, MF, and RND transporters (19). Thus, it is not surprising that TolC has been implicated in the virulence of multiple human pathogens, including *E. coli* (37, 38), *Salmonella* (39), *Haemophilus* (40), *Francisella* (41, 42), *Legionella* (43), and *Brucella* (44). Consistent with its function as the outer membrane pore for multiple different transport systems, the contribution of TolC to pathogenesis is often pleiotropic. For example, TolC has frequently been linked to virulence in enteric pathogens, including in *V. cholerae* (21), due to its contribution to antimicrobial resistance. In addition, TolC is required for the secretion of virulence factors by type I secretion systems, including hemolysin (45) and enterotoxin (46) in *E. coli* and MARTX toxin in *V. cholerae* (21). However, studies linking TolC to the expression of virulence genes have been limited

to *Salmonella* (47) and *V. cholerae* (described further below). However, the fact that many TolC-dependent transporters have been linked to virulence gene expression suggests that *tolC* has more extensive contributions to virulence gene expression than are currently appreciated (12, 36, 48–51).

The presented studies document the requirement for *tolC* in *V. cholerae* virulence factor production, but the mechanism linking *tolC* to *leuO* induction remains unclear. Recent studies defined a metabolic feedback regulatory loop in RND efflux-impaired *V. cholerae* (12, 52). These studies indicated that metabolites, which were substrates for the RND efflux systems, accumulated intracellularly in the absence of RND-mediated efflux. The accumulating metabolites then activated periplasmic sensors to affect transcriptional responses. This included *leuO*-dependent virulence repression via *aphA*, as observed here. Based on this, we propose that virulence repression in the Δ*tolC* mutant also resulted from the intracellular accumulation of metabolites that led to increased *leuO* transcription and the subsequent downregulation of the ToxR regulon (Fig. 6). The fact that deletion of *leuO* in the Δ*tolC* strain restored virulence factor production (Fig. 3C) and that a Δ*tolC* mutant phenocopied an RND efflux null mutant (Fig. 5B and C) provide support for this model. The chemical stimuli that were responsible for activating *leuO* in the RND impaired strains was not determined, but several metabolites that have been shown to activate *leuO* transcription are substrates of the RND efflux systems (e.g., indole, bile salts, cyclic dipeptides, and products of intermediate and central metabolism) and, thus, could contribute to the phenotype observed here (11, 24, 53). The fact that the *V. cholerae* Δ*tolC* strain phenocopies the antimicrobial susceptibility profile of an isogenic RND efflux-negative mutant (21, 34), combined with the feedback effects of *tolC* deletion on RND efflux system expression (Fig. 5), provide additional support for this model. Whether similar feedback regulatory mechanisms occur in other pathogens where *tolC* has been linked to virulence gene expression remains to be investigated.

There is also evidence to suggest that TolC functions in metabolite feedback regulation in *V. cholerae* strains belonging to the classical biotype. Classical biotype strains are believed to be responsible for pandemic cholera prior to 1961, when the seventh pandemic began with the emergence of the El Tor biotype (1, 54). El Tor biotype strains have since displaced the classical biotype in causing epidemic and pandemic disease. The two biotypes are differentiated by multiple phenotypes, including the conditions required for ToxR regulon activation. El Tor strains require AKI growth conditions to activate the ToxR regulon, whereas classical strains express the ToxR regulon in LB broth, pH 6.8, at 30°C and in minimal medium supplemented with asparagine, arginine, glutamic acid, and serine (NRES). Interestingly, deletion of *tolC* in a classical *V. cholerae* strain resulted in increased *toxT* expression under noninducing conditions (i.e., minimal medium lacking NRES) (55). The mechanism responsible for increased *toxT* transcription was not determined but was attributed to feedback regulation through ToxR or TcpP in response to the intracellular accumulation of efflux-dependent metabolic byproducts. These results, while contrary to the regulatory effects of *tolC* mutation observed in El Tor biotypes, further support the conclusion that *tolC* affects gene expression via metabolite feedback processes that are likely linked to TolC's function as the outer membrane pore protein for multiple transport systems.

While the data presented here document the effects of *tolC* on virulence gene expression, we note that TolC also affects other aspects of *V. cholerae* virulence. For example, previous studies have documented that *V. cholerae tolC* is required for secretion of MARTX toxin. MARTX toxin has been shown to target neutrophils to prevent clearance of *V. cholerae* in the gut (21, 56). Whether *tolC* is involved in the secretion of other proteins and whether *tolC* can affect virulence by other posttranscriptional mechanisms is unclear. We noted that deletion of *tolC* in *V. cholerae* O1 El Tor strain C6706 did not affect the production of the hemagglutinin (HA)/protease (data not shown), suggesting that it does not contribute to HA protease secretion (57).

In summary, the results presented here expand the function of TolC in *V. cholerae* pathogenesis and confirm that TolC makes pleiotropic contributions to *V. cholerae*

pathogenesis. Given the conserved requirement for TolC in pathogenesis and antimicrobial resistance among Gram-negative pathogens, TolC is a viable target for the development of novel therapeutics that could inhibit virulence and/or potentiate antibiotics.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *V. cholerae* O1 El Tor strain N16961 was acquired from our laboratory storage. N16961 JB58 (Δ*lacZ* Sm^r) and JB58Δ*tolC* strains were previously described (21). JB58 was used as the wild-type strain in this study. All *lux* reporter strains were constructed in our laboratory. *E. coli* strain EC100λpir (Epicentre, Madison, WI, USA) was used as the host strain for DNA cloning experiments. *V. cholerae* and *E. coli* strains were routinely grown in lysogeny broth (LB) medium or on LB agar plates at 37°C. *In vitro* virulence gene-inducing conditions for *V. cholerae* (i.e., AKI conditions) were performed by diluting overnight LB medium cultured strains (10^{-4}) into 150- by 15-mm borosilicate glass test tubes containing 10 ml of AKI broth (4 g Difco yeast extract, 15 g Bacto peptone, and 5 g NaCl per liter, pH 7.4). This was followed by static incubation for 4 h at 37°C or until the optical density at 600 nm was greater than 0.08. Thereafter the cultures were transferred to 125-ml Erlenmeyer flasks and then incubated for 1 h or overnight at 37°C with shaking. Carbenicillin and streptomycin were added to the growth medium at 100 μg/ml when needed. Strains bearing the pBAD18 expression vector were cultured with the addition of 0.1% L-arabinose to induce expression from the arabinose-regulated promoter when necessary.

Chemicals and reagents. Chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Carbenicillin and streptomycin stock solutions were made in ultrapure water, filter sterilized, aliquoted, and stored at −20°C until needed. Oligonucleotides were designed based on the *V. cholerae* N16961 and C6706 genomes (25, 58) and purchased from Integrated DNA Technologies (Coralville, IA, USA), and enzymes for cloning were purchased from New England Biolabs (Beverly, MA, USA) (25).

Strain construction. Strains, plasmids, and oligonucleotide primers are listed in Table 1. The construction of the Δ*tolC* mutant of *V. cholerae* strain N16961 has been previously reported (21). The Δ*tolC* Δ*leuO* and ΔRND Δ*tolC* strains were generated by allelic exchange using the pWM91Δ*leuO* and pWM91Δ*tolC* suicide vectors as previously described (21, 24). The construction of the *lux*-based reporter plasmids for the ToxR regulon genes in pTB18 has been described (52). Reporter plasmids were introduced into the *V. cholerae* strains by electroporation or conjugation from *E. coli* strain SM10λ*pir*.

Virulence factor production. Virulence factor production was assessed by quantifying cholera toxin using a GM1 enzyme-linked immunosorbent assay (ELISA) in the indicated strains following overnight growth under AKI conditions in AKI medium as previously described (59). Purified cholera toxin was used as a standard for quantitation. TcpA production was assessed by Western blotting using polyclonal antibodies to TcpA as previously described (24).

Porin production. Whole-cell lysates were prepared from the indicated *V. cholerae* strains following growth under AKI conditions for 5 or 18 h. Culture aliquots were collected from the test strains, normalized by the optical density (OD) at 600 nm before being solubilized in sample buffer. The whole-cell lysates were then separated by SDS-PAGE on a 12% polyacrylamide gel and stained with Coomassie blue for visualization. WT and Δ*toxRS* strains were included as controls to demark the OmpU and OmpT bands, respectively.

Transcriptional reporter assays. *V. cholerae* N16961 strain JB58 and its isogenic Δ*tolC* mutant containing the indicated lux reporter plasmids were cultured under AKI growth conditions in AKI medium. At the indicated time points, culture aliquots (200 μl) were collected in triplicate and transferred to the wells of a 96-well white microtiter plate with a clear bottom. Luminescence production and the optical density at 600 nm were determined on a BioTek Synergy HT plate reader. The results were reported as the mean ± standard deviation number of relative luminescence units (RLU) divided by the optical density from three independent biological replicates.

***Ex vivo* virulence model.** The *ex vivo* virulence gene induction model was performed essentially as described previously (35). Briefly, fresh porcine small intestines were procured from the University of Pittsburgh Division of Animal Resources. The intestines were derived from discarded tissues of freshly euthanized animals. The intestines were splayed open, and 1-cm-square sections of the intestine were excised. The intestinal sections were placed into a petri dish, and 20 μl of *V. cholerae* inoculum was placed on the lumen side (faced up). The inoculum was prepared as follows. Overnight cultures grown in LB medium were diluted 1:100 into 3 ml of fresh LB medium and incubated with shaking at 37°C until they reached an optical density of ~1.0, and then a 1-ml aliquot was collected from each culture, washed in an equal amount of minimum essential medium (MEM) twice before being resuspended in 500 μl of MEM to generate the inoculum. After inoculation, the covered petri dishes were incubated at 37°C in a sealed anaerobic jar containing a Mitsubishi AnaeroPack-Anaero to generate anaerobic conditions. After 4 to 5 h, the petri dishes were imaged using an IVIS Lumina X5 imaging system (Perkin Elmer), and luminescence production on the captured images was quantified using the Living Image software (Perkin Elmer).

Quantitative real-time PCR. Total RNA was isolated from cultures of *V. cholerae* strains after 5 h of growth under AKI conditions in AKI medium using TRIzol as described by the manufacturer's directions. cDNA was generated with the total RNA using Superscript III RT (Invitrogen). The expression level of specific genes was quantified by amplifying about 25 ng cDNA with 0.5 μM primers using SYBR green PCR mix (Fisher Scientific) in a Step One Plus real-time PCR system machine (Applied Biosystems). DNA

gyrase (*gyrA*) was used as an internal control. Changes in gene expression were calculated by the $2^{-\Delta\Delta CT}$ method and are presented as means ± standard errors from three biological replicates, with each biological replicate generated from three technical replicates.

Statistical methods. Statistical analyses of the data were performed using GraphPad Prism software (GraphPad Software). The specific statistical method used for each data set is described in the figure legend.

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