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MOLECULAR PATHOGENESIS

Indole Inhibits ToxR Regulon Expression in *Vibrio cholerae*

Mondraya F. Howard,[a] X. Renee Bina,[a] James E. Bina[a]

[a]Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA

ABSTRACT Indole is a degradation product of tryptophan that functions as a signaling molecule in many bacteria. This includes *Vibrio cholerae*, where indole was shown to regulate biofilm and type VI secretion in nontoxigenic environmental isolates. Indole is also produced by toxigenic *V. cholerae* strains in the human intestine, but its significance in the host is unknown. We investigated the effects of indole on toxigenic *V. cholerae* O1 El Tor during growth under virulence inducing conditions. The indole transcriptome was defined by RNA sequencing and showed widespread changes in the expression of genes involved in metabolism, biofilm production, and virulence factor production. In contrast, genes involved in type VI secretion were not affected by indole. We subsequently found that indole repressed genes involved in *V. cholerae* pathogenesis, including the ToxR virulence regulon. Consistent with this, indole inhibited cholera toxin and toxin-coregulated pilus production in a dose-dependent manner. The effects of indole on virulence factor production and biofilm were linked to ToxR and the ToxR-dependent regulator LeuO. The expression of *leuO* was increased by exogenous indole and linked to repression of the ToxR virulence regulon. This process was dependent on the ToxR periplasmic domain, suggesting that indole was a ToxR agonist. This conclusion was further supported by results showing that the ToxR periplasmic domain contributed to indole-mediated increased biofilm production. Collectively, our results suggest that indole may be a niche-specific cue that can function as a ToxR agonist to modulate virulence gene expression and biofilm production in *V. cholerae*.

KEYWORDS cholera, indole, biofilms, virulence regulation

Vibrio cholerae is a Gram-negative bacterial pathogen and the causative agent of the acute diarrheal disease cholera. *V. cholerae* is an inhabitant of aquatic ecosystems and is acquired by ingestion of *V. cholerae*-contaminated food or water. After ingestion, *V. cholerae* colonizes the small intestine where it produces virulence factors, including toxin-coregulated pilus (TCP) and cholera toxin (CT), that result in the production of a severe secretory diarrhea. The expression of these virulence genes is under the control of ToxR, a membrane-bound regulatory protein that functions as the masthead of the ToxR virulence regulon.

The ToxR regulon is a hierarchal regulatory system that activates virulence gene expression following host entry. The *in vivo* stimuli that activate the ToxR regulon are unclear, but *in vitro* it has been shown to be modulated by a wide variety of environmental cues, including temperature, pH, osmolarity, oxygen, and bile salts (1–4). The induction of the regulon begins with two cytoplasmic proteins, AphA and AphB, which activate expression of the *tcpPH* operon (5). TcpP then binds with ToxR at the *toxT* promoter to initiate *toxT* expression (6). Thereafter, ToxT directly activates expression of the *ctxAB* and *tcpABQCRDSTEF* operons, which encode CT and TCP, respectively. Once *V. cholerae* is established in the small intestine, it replicates to high cell density before disseminating in the secretory diarrhea. Although much has been discerned about the transition of *V. cholerae* from the environment into the gastrointestinal tract, little is known about *V. cholerae* exiting the host. Several studies have documented a pheno-

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Address correspondence to James E. Bina, jbina@pitt.edu.

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typic shift that occurs late during infection that is characterized by a downregulation of virulence genes and upregulation of genes that contribute to both a hyperinfectious phenotype and enhanced fitness in aquatic environments (7, 8). These late infection phenotypes are likely critical for the life cycle of toxigenic *V. cholerae*, contributing both to epidemic spread and to survival and persistence in aquatic ecosystems.

Recent studies suggest that ToxR responds to extracellular cues to regulate the expression of genes that are important for adaptation to growth at high cell density. Given that *V. cholerae* replicates to high cell density during human infection, these results suggest that ToxR could contribute to the expression of genes during late infection. This conclusion was supported by the observation that the cyclic dipeptide metabolite cyclo(Phe-Pro) (cFP) accumulates to high concentrations at the stationary phase in the culture supernatants of some *V. cholerae* strains (9). cFP has been shown to signal through the periplasmic domain of ToxR to activate the expression of the LysR-family transcription factor *leuO* (10). LeuO then contributes to diverse phenotypes that are associated with late infection, including regulating genes involved in dissemination and transmission (e.g., biofilm, acid tolerance, antimicrobial resistance, and CT/TCP production) (10–14). The fact that *leuO* is positively regulated by ToxR (12) and maximally expressed at stationary phase (10) is consistent with this hypothesis. In addition, the requirement for ToxR in biofilm production (15), a phenotype that is associated with human shed *V. cholerae* (16), further bolsters this idea.

ToxR is a membrane-associated protein that contains a periplasmic sensing domain that is linked to a cytoplasmic DNA binding domain by a single transmembrane-spanning domain (17). The activity of ToxR is enhanced by interaction with another membrane-associated protein, ToxS (18). ToxR is thought to function as an environmental sensor that transduces environmental cues into transcriptional responses (3). There is accumulating evidence suggesting that low-molecular-weight compounds, both endogenously produced and exogenous, influence ToxR activity via interaction with its periplasmic domain (PPD) (10, 12, 19). Genetic studies from our laboratory showed that ToxR-dependent induction of *leuO* and *ompU* in response to cyclic dipeptides and bile salts was dependent on the presence of the PPD (10, 12), but the mechanism by which they activated ToxR was unclear. However, recent biochemical studies put forth a new model that can explain our genetic results (19). These studies showed that ToxR agonists bind to and destabilize the ToxR PPD. The destabilization of the PPD facilitates the formation of heterodimers between the ToxR PPD and the periplasmic domain of ToxS, which results in ToxR activation. Altogether, these findings support the conclusion that ToxR is an environmental sensor that responds to environmental cues via its PPD to effect the expression of adaptive responses.

Resistance-nodulation-division (RND) efflux systems are ubiquitous transporters in Gram-negative bacteria. The RND systems are critical for multiple antibiotic resistance due to their broad substrate specificity, which provides cross-resistance to multiple antimicrobial compounds (20). However, the RND systems also affect diverse phenotypes in bacteria, which suggests that they contribute to unknown physiological functions. This is true in *V. cholerae*, where RND-mediated efflux is required for virulence gene expression and cell homeostasis (21, 22). Recently, we documented that the *V. cholerae* RND efflux systems were linked to the ToxR-dependent regulation of virulence factor production (23). We found that in the absence of RND-mediated efflux, *leuO* transcription increased and led to repression of the ToxR regulon. This suggested a model whereby ToxR downregulated virulence genes in response to the accumulation of unknown endogenous metabolites that are normally exported from the cell by RND transporters.

The link between RND efflux and CT and TCP production suggested that products of *V. cholerae* metabolism may function in a feedback circuit to regulate virulence. Although the native substrates of the RND efflux systems are largely unknown, previous studies suggested that indole was a substrate for the *V. cholerae* VexAB RND efflux system (24). Indole is a bioactive metabolite produced by many bacteria, including *V. cholerae*. Indole has been shown to affect diverse bacterial phenotypes (25) and has

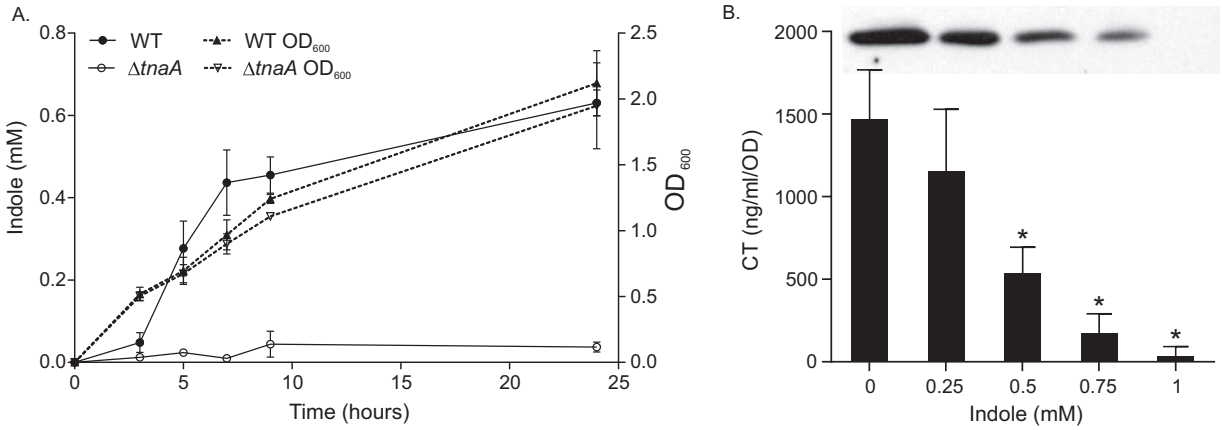

FIG 1 Indole is produced at stationary phase and inhibits virulence factor production. (A) WT strain JB58 and an isogenic Δ*tnaA* mutant were cultured in LB medium at 37°C with shaking. Culture aliquots were collected at the indicated times; cell growth was assessed (right axis, triangle symbols), and indole production was quantified (left axis, circle symbols). The presented data show the means ± the standard deviations (SD) for at least three independent experiments. (B) *V. cholerae* strain JB58 was grown under AKI conditions with the indicated amounts of indole before CT and TcpA production (inset) were assayed. The CT data are the means ± the SD of three independent experiments. Statistical significance was determined using one-way analysis of variance (ANOVA) comparing each mean to that of the methanol control (*, $P < 0.05$).

been proposed to function as an interkingdom signaling molecule. Indole is produced in the human gut by the resident flora and is thought to enhance the barrier functions of the intestinal epithelium (26–28). *V. cholerae* also produces large amounts of indole during human infection (29). In *V. cholerae* indole is generated during stationary phase from the breakdown of tryptophan by the enzyme tryptophanase (*tnaA*). In environmental strains of *V. cholerae*, exogenous indole was shown to promote biofilm production in addition to affecting the expression of other genes (30). The observations that indole is produced during *V. cholerae* infection of humans and that biofilm contributes to hyperinfectivity and environmental survival (16, 31) suggest the possibility that indole may function as a signal *in vivo* that influences gene expression during infection.

We examined here the effect of indole on toxigenic *V. cholerae*. We found that indole was produced by a *tnaA*-dependent process and accumulated to a maximum concentration of ~0.6 mM in the extracellular supernatant at early stationary phase. RNA sequencing of *V. cholerae* exposed to indole revealed that indole affected diverse physiological processes, including the activation of genes involved in biofilm production and the repression of genes involved in virulence. Subsequent analysis showed that indole-dependent activation of ToxR resulted in increased *leuO* expression. The upregulation of *leuO* contributed to indole-mediated repression of the ToxR regulon and increased biofilm formation. Altogether, our findings suggest that indole may function as an environmental cue during the life cycle of toxigenic *V. cholerae*.

RESULTS

Indole production. Indole production during *V. cholerae* human infection was first reported in the late 1800s and has been used as a diagnostic (29). To confirm that the O1 El Tor strain N16961 produced indole, we cultured strain JB58 and an isogenic *tnaA* deletion mutant in Luria-Bertani (LB) broth and quantified indole production over time. The results showed that indole production began at mid-log phase and reached the maximal concentration of ~0.6 mM in stationary phase (Fig. 1A). These results are similar to findings reported for both environmental *V. cholerae* (30) and other O1 strains (32). Similar levels of indole production were also reported among the *Enterobacteriaceae* (33, 34). Based on these results, we selected 0.5 mM indole as a physiologically relevant concentration to assess the effects of indole on *V. cholerae*. We noted that indole has been reported to be toxic at high concentrations (35), but 0.5 mM indole does not produce deleterious effects on *V. cholerae* (30).

TABLE 1 Functional categories of indole-responsive genes

Functional group	No. of genes		
	Upregulated	Downregulated	Total
Amino acid biosynthesis	4	0	4
Biosynthesis of cofactors, prosthetic groups, and carriers	0	1	1
Cell envelope	6	2	8
Cellular processes	2	1	3
Conserved, hypothetical, and unknown	23	22	45
DNA replication, recombination, and repair	0	1	1
Metabolism	21	2	23
Pathogenesis	2	21	23
Purines, pyrimidines, nucleosides, and nucleotides	1	0	1
Protein fate	0	5	5
Regulatory functions	3	6	9
Transport and binding proteins	12	8	20
Total	74	69	143

Indole inhibits CT and TcpA production. The observation that *V. cholerae* produced indole at a high cell density suggested that indole could be an environmental cue that contributed to virulence repression during infection. To test this, we quantified the effects of indole on CT and TCP production by culturing wild-type (WT) strain JB58 under AKI conditions in the presence of increasing concentrations of indole (Fig. 1B). The results showed an indole dose-dependent decrease in CT and TCP production beginning at 0.25 mM indole. CT production was reduced by ~65% at 0.5 mM and ~80% at 0.75 mM. CT and TcpA production was abolished at 1 mM indole. From these results, we concluded that indole was a virulence inhibitor.

Determination of the indole transcriptome. Since the results presented above suggested that indole was a virulence inhibitor, we sought to determine the mechanism by which indole inhibited CT and TCP production. We therefore performed RNA sequencing (RNA-seq) on *V. cholerae* during growth under virulence gene inducing conditions in the presence or absence 0.5 mM indole. This identified 143 differentially expressed genes: 74 upregulated genes and 69 downregulated genes (Table S2). The largest category of differentially regulated genes was those of unknown function (45 genes), followed by metabolism (23 genes), pathogenesis (23 genes), and transport and binding (20 genes) (Table 1).

Consistent with these results, the genes involved in CT and TCP production were largely downregulated by indole (i.e., *ctxAB* and *tcpABQCRDSTEF* operons; Table S2). Among the regulatory genes in the ToxR virulence regulon, *toxT* was identified as being repressed, suggesting that indole affected *toxT* transcription. We suspect that we did not identify genes that are upstream of *toxT* as being differentially expressed because the ToxR regulon had not been fully induced due to the early time point used for RNA collection. Interestingly, the genes involved in the production of multifunctional-auto processing repeats-in-toxin (MARTX) were also repressed by indole. The mechanisms regulating MARTX are unknown but MARTX was previously shown to be regulated according to growth (36, 37), suggesting that indole could play a role in MARTX expression. Collectively, these results confirmed the CT/TCP bioassays and suggested that indole affects the expression of the ToxR regulon.

Previous studies using nontoxigenic environmental *V. cholerae* isolates showed that indole upregulated the expression of genes involved in biofilm production and production of the type VI secretion system (T6SS) (30). Consistent with these studies, we also observed that indole increased the expression of genes involved in biofilm production (Table S2). Vibrio polysaccharide (VPS) is the major carbohydrate component of *V. cholerae* biofilm. The genes involved in VPS production, the *vps* genes, are clustered in two loci, *vpsABCDEFGHIJK* (VCO917-VC0927) and *vpsLMNOPQ* (VC0934-VC0939) (38), and several genes within each of these two clusters (*vpsU*, *vpsA-vpsB*, and *vpsL-vpsN*) were upregulated in the presence of indole. In addition, the expression of

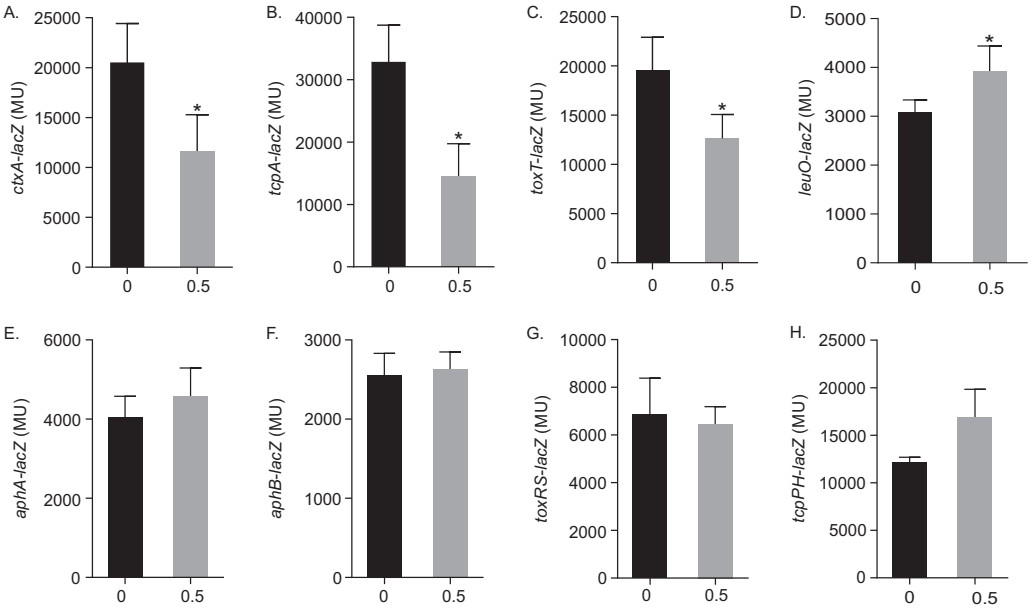

FIG 2 Indole represses *toxT* expression. (A to H) *V. cholerae* JB58 strains carrying the indicated transcriptional reporters were cultured under AKI conditions for 5 h in the presence or absence of 0.5 mM indole, and the gene expression was quantified. The presented results are means ± the SD for at least three independent experiments. Statistical significance was determined using a *t* test comparing each mean to the no-indole control (*, $P < 0.05$). MU, Miller units.

the genes encoding the biofilm matrix proteins Bap1, RbmA and RbmC were increased by indole (39). We did not observe changes in the expression of genes involved in type VI secretion. This is in contrast to what was observed in environmental *V. cholerae* isolates (30). The reasons for this are unknown, but it may be due to strain variation since toxigenic *V. cholerae* strains, such as N16961 used in this study, do not typically express the type VI secretion systems *in vitro* (40).

Indole represses *toxT* expression. The RNA-seq data suggested that indole inhibited CT and TCP production by repressing the ToxR regulon. Since the ToxR regulon is a hierarchical regulatory system, we sought to determine at which point indole was acting in the ToxR regulon. To test this, we cultured WT strain JB58 carrying *lacZ* transcriptional reporters for each of the regulatory genes in the ToxR regulon under AKI conditions in the presence or absence of 0.5 mM indole and quantified reporter expression as β-galactosidase activity. We first examined the effect of indole on *ctxAB-lacZ* and *tcpA-lacZ* expression (Fig. 2A and B). We observed that the addition of indole resulted in 1.7- and 2-fold reductions in *ctxA* and *tcpA* promoter activities, respectively. This finding was consistent with the CT and TcpA results presented in Fig. 1. Since *ctxA* and *tcpA* promoters are directly activated by ToxT, we tested whether indole affected *toxT* expression. Consistent with the RNA-seq results, the presence of indole reduced *toxT* expression by ~1.5-fold (Fig. 2C). We next tested the genes that are upstream of *toxT* in the ToxR regulon. The results showed that the expression of *aphA*, *aphB*, *tcpP*, and *toxR* was unchanged in the presence of 0.5 mM indole (Fig. 2E to H). The expression of *tcpP* appeared to slightly increase in the presence of indole, but the increase was not significant. Overall, these results indicate that indole inhibited CT and TCP production by repressing *toxT* transcription.

LeuO contributes to virulence repression by indole. The results presented above suggested that indole repressed *toxT* transcription, but not *aphA*, *aphB*, *tcpPH*, or *toxRS* transcription. This suggested that indole may be functioning through another regulator. The RNA-seq results identified nine regulatory genes as being differentially expressed in indole (Table 1), including the LysR-family transcription factor *leuO*, which was upregulated 1.5-fold in the presence of indole (Table S2). LeuO is a global regulator that has been linked to multiple *V. cholerae* phenotypes, including ToxR regulon

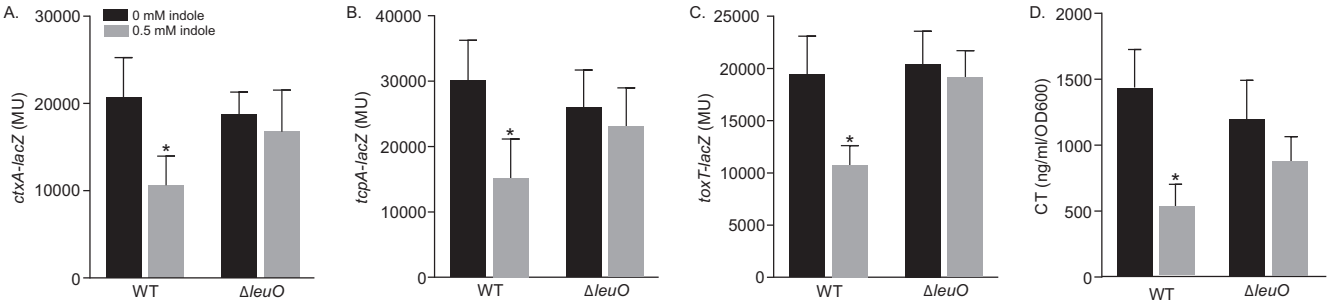

FIG 3 LeuO contributes to indole-dependent virulence repression. *V. cholerae* strain JB58 and an isogenic *leuO* deletion strain carrying (A) *ctxA-lacZ*, (B) *tcpA-lacZ*, or (C) *toxT-lacZ* reporters were cultured under AKI conditions for 5 h in the presence or absence of 0.5 mM indole, and gene expression was quantified by determining the β-galactosidase activity. (D) WT and an isogenic Δ*leuO* mutant were cultured overnight in AKI medium supplemented with 0 or 0.5 mM indole before CT production was quantified by GM1 ELISA. The data are presented as means ± the SD for three independent experiments. Statistical significance was determined using two-way ANOVA, comparing each mean to the methanol control (*, $P \leq 0.05$).

repression (10, 12, 13). We therefore investigated whether LeuO contributed to the indole-dependent virulence repression. We first confirmed that indole increased *leuO* transcription by comparing *leuO-lacZ* expression in WT strain JB58 during growth under AKI conditions in the presence or absence of 0.5 mM indole. The results showed that *leuO* expression was significantly increased with the addition of indole (Fig. 2D), confirming the RNA-seq results.

Expression of *leuO* has been correlated with downregulation of the ToxR regulon (10, 23). Therefore, we examined if LeuO contributed to the indole-dependent repression of the ToxR regulon. To test this, we compared *toxT-lacZ*, *ctxA-lacZ*, and *tcpA-lacZ* expression in the WT and in an isogenic Δ*leuO* mutant during growth under AKI conditions in the presence or absence of indole. The results showed that indole caused an ~2-fold decrease in the expression of *toxT*, *ctxA*, and *tcpA* in the WT, whereas the negative effects of indole on the expression of all three genes were abated in the *leuO*-negative strain (Fig. 3A to C). These results suggested that LeuO contributed to the indole-dependent inhibition of virulence gene expression.

We hypothesized that if indole inhibited CT and TCP production via LeuO, then deletion of *leuO* would restore virulence factor production in cells grown in the presence of indole. We tested this by quantifying CT production in WT and Δ*leuO* strains during growth under AKI conditions in the presence of 0.5 mM indole. The results showed that the presence of indole resulted in ~60% reduction in CT production in the WT. In contrast, the negative effects of indole on CT production were partially suppressed in the Δ*leuO* mutant (Fig. 3D). The fact that *leuO* deletion did not completely restore CT production in the presence of indole suggests that indole has additional effects on virulence factor production independent of LeuO.

Indole is a ToxR agonist. The finding that indole activated *leuO* transcription led us to hypothesize that indole was a ToxR agonist (41–43). To test this, we examined the effect of indole on OmpU production in WT strain JB58 during growth in minimal T medium. The amino acid cocktail NRES was included as a negative control (NRES promotes OmpU production by activating *toxR* transcription [44], not by altering ToxR activity). The results showed that indole increased OmpU production in a dose-dependent manner (Fig. 4A). To discriminate if the increase in OmpU was due to activation of preformed ToxR or due to increased ToxR production, we performed ToxR Western blotting (Fig. 4A). The results showed that indole did not affect ToxR abundance, whereas NRES supplementation increased ToxR and OmpU production as previously reported (44). Consistent with this, exogenous indole also did not affect *toxR* transcription (Fig. 4B). Taken together, these results suggested that indole was a ToxR agonist that increased OmpU production by enhancing the activity of preformed ToxR.

The ToxR PPD is required for agonist-dependent activation of *leuO* transcription (10, 12, 23). Therefore, we tested whether the ToxR PPD was required for indole-dependent *leuO*

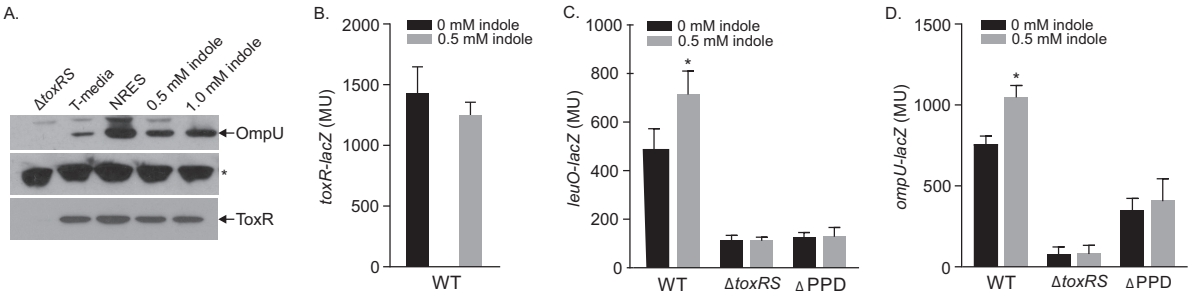

FIG 4 Indole is an agonist of ToxR activity. (A) ToxR and OmpU Western blots. WT strain JB58 was cultured in T medium supplemented with amino acids (NRES) or increasing concentrations of indole for 4 h, and culture aliquots were collected, normalized by determining the OD_{600}, and used for Western blotting with an anti-ToxR (bottom) or an anti-OmpU (top and middle) polyclonal antibody. A Δ*toxRS* mutant grown in T medium was included as a marker for ToxR and OmpU. The asterisk indicates a nonspecific band that was used as a loading control. (B) Indole does not affect *toxR* expression. WT strain JB58 carrying a *toxRS-lacZ* reporter was cultured in T medium with the indicated indole concentrations for 4 h when β-galactosidase was quantified. The data are means ± the SD for three independent experiments. (C and D) Indole increases the expression of ToxR-dependent genes. The indicated *V. cholerae* strains harboring *leuO-lacZ* or *ompU-lacZ* reporters were cultured under the conditions described above, and gene expression was quantified by determining the β-galactosidase activity. The data are means ± the SD for at least three independent experiments. Statistical significance was determined by two-way ANOVA (*, $P \leq 0.05$ relative to the methanol control).

induction. To do this, we cultured the WT strain JB58 and the isogenic Δ*toxRS* (DT733) and *toxR*$^{\Delta PPD}$ (SS4) strains in minimal T medium containing 0.5 mM indole for 4 h. We then quantified *leuO-lacZ* and *ompU-lacZ* expression. The results showed that indole supplementation increased *leuO* and *ompU* expression ~1.5-fold (Fig. 4C and D) and that the expression of both genes was abrogated in the *toxR*$^{\Delta PPD}$ mutant. From this, we concluded that indole activity was dependent on the periplasmic domain of ToxR. Since the addition of indole to T medium did not affect *toxR* expression (Fig. 4B), these results suggested that exogenous indole activated ToxR by a process that was dependent on its periplasmic sensing domain.

ToxR and LeuO mediate indole-dependent biofilm production. Indole was previously shown to activate biofilm formation in environmental *V. cholerae* isolates (30). This phenotype appears to be conserved in toxigenic *V. cholerae*, as evidenced by the indole-dependent increase in transcription of *vps* and biofilm matrix genes in the RNA-seq results (Table S2). Since LeuO and ToxR were shown to contribute to biofilm formation (14, 45), we examined whether they contributed to indole-dependent biofilm activation. To test this, we compared the effects of indole on biofilm production in WT and Δ*leuO*, Δ*toxRS*, and Δ*tnaA* mutant strains (Fig. 5). The results showed that indole increased biofilm formation in WT relative to the no indole control. Biofilm production in an indole-negative Δ*tnaA* mutant was reduced relative to the WT strain, suggesting that endogenous indole production functions in a feedback loop to enhance biofilm production. The biofilm defect associated with the loss of *tnaA* was complemented by adding indole back to the growth medium, confirming previous results (30).

In contrast, the Δ*toxRS* mutant displayed a biofilm defect regardless of culture conditions, confirming the requirement for ToxR in biofilm production (19). Biofilm production in the Δ*leuO* mutant was reduced compared to the WT, and indole addition partially restored biofilm production (relative to the WT plus indole). Since the ToxR PPD was required for indole-dependent *leuO* induction (Fig. 4C), we tested whether the PPD was also required for biofilm production. Therefore, we assayed biofilm production in a *toxR*$^{\Delta PPD}$ mutant. In the absence of indole the *toxR*$^{\Delta PPD}$ mutant, in which *leuO* expression is impaired (23), produced similar amounts of biofilm as the Δ*leuO* mutant. However, in contrast to the *leuO* mutant, the addition of exogenous indole did not restore biofilm production in the *toxR*$^{\Delta PPD}$ mutant (Fig. 5). From these results, we concluded that LeuO and the periplasmic signal-sensing domain of ToxR contribute to indole-dependent regulation of biofilm formation.

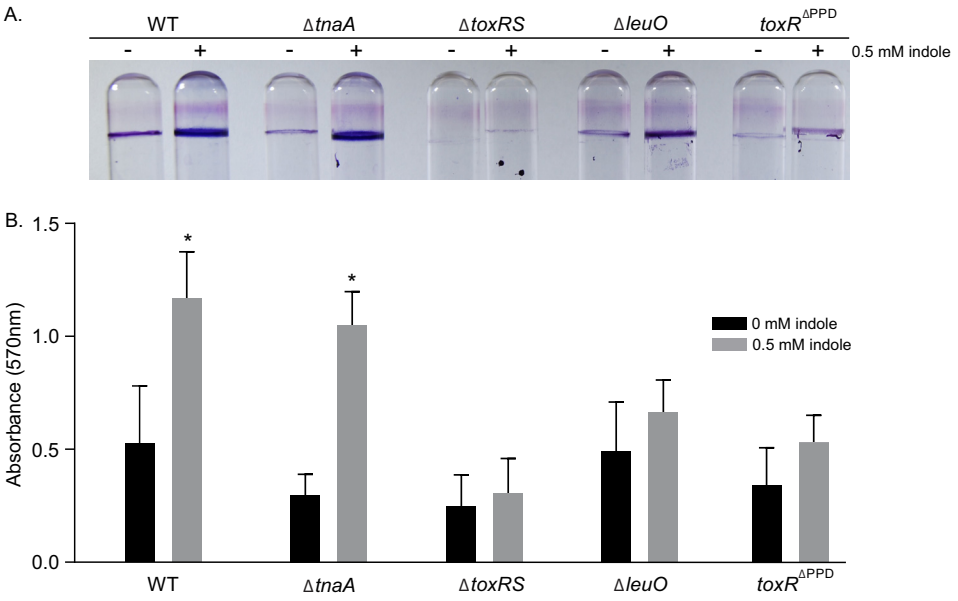

FIG 5 Effects of indole on biofilm formation. Biofilm assays were performed with WT strain JB58 and the indicated isogenic mutant strains in the presence or absence of indole. Crystal violet-stained borosilicate tubes (A) and quantifications of the acetic acid-solubilized dye (B) are shown for the indicated strains after 48 h of static growth in LB medium supplemented with 0 or 0.5 mM indole at room temperature. The stained test tubes are representative of at least three independent experiments, with each assay being performed in triplicate. The absorbance data are presented as means ± the SD for three experiments. Statistical significance was determined using two-way ANOVA (*, $P \leq 0.05$ relative to the methanol control).

DISCUSSION

Indole is a bioactive molecule that is widespread in nature and thought to be an intraspecies, interspecies and interkingdom signaling molecule. Mammals do not produce indole, but indole is present at high concentrations in their gut, being produced by the resident flora. *V. cholerae* also produces indole during human infection (29), and as in most organisms, the function of indole in *V. cholerae* biology is unclear. Here, we document that indole has broad physiological effects on *V. cholerae* and suggest that indole may function as a niche-specific signaling molecule in *V. cholerae*.

Indole is produced through the degradation of tryptophan by tryptophanase (TnaA). The *tnaA* gene is regulated by catabolite repression in the *Enterobacteriaceae* (46), making it predominantly expressed at the stationary phase. Consistent with this, we observed maximal indole production in *V. cholerae* during the transition to stationary phase, where the maximal indole concentration reached ~0.6 mM (Fig. 1). These results mirror previous studies with environmental *V. cholerae* isolates (30). We did not observe indole production from *V. cholerae* cultured under virulence-inducing conditions in AKI medium. Since indole production is dependent on exogenous tryptophan (47), we speculate that this was due to low levels of tryptophan in AKI medium. This conclusion was supported by the observation that tryptophan addition to AKI resulted in indole production (data not shown). The production of indole appears to be relevant *in vivo* as indole is present in cholera patients (29). Consistent with *in vivo* indole production, *tnaA* expression was also upregulated in infant rabbit and infant mouse colonization models (48). The latter findings suggest that the results reported in this study may be relevant during *V. cholerae* growth *in vivo*.

V. cholerae shed from animal and human hosts is primed for survival in aquatic ecosystems and for the infection of new hosts (8, 49). However, the factors that contribute to the development of these phenotypes are not known. Transcriptional profiling of cholera stool samples revealed a physiological state characterized by increased expression of genes involved in amino acid biosynthesis, nutrient transport, and metabolism (7, 50). Here, we observed that indole regulated similar functional

groups. Indole activated the expression of genes involved in tryptophan biosynthesis and acquisition of carbon sources such as sialic acid (VC1779, or *siaP*) and glycerol (VCA0137, or *glpT*). In aquatic environments, *V. cholerae* faces nutrient limitation; thus, indole-dependent induction of these genes may facilitate storage of nutrients to survive under nutrient-limiting conditions. Notable genes activated by indole included glycerol kinase *glpK* (VCA0774), the cadaverine/lysine transporter *cadB* (VC0280), and chitin binding protein *gbpA* (VCA0811). Expression of *glpK* is increased during late infection and is important for *V. cholerae* persistence in pond water (8). CadB functions in *V. cholerae* acid tolerance response, an adaptive phenotype that was reported to increase *V. cholerae* infectivity (51). GbpA is a colonization factor that was reported to mediate adherence to human intestinal cells and chitinous organisms in aquatic environments (52, 53). Overall, the transcriptional results are consistent with indole impacting physiological processes that have been observed during late infection.

Transcriptional profiling of cholera stool samples also revealed the repression of the ToxR virulence regulon (7, 54). Here, we propose that indole repressed virulence gene expression by two independent mechanisms that converged on *toxT*. The first mechanism was dependent on ToxR activation of *leuO* transcription. ToxR positively regulates *leuO* expression in response to environmental stimuli (e.g., bile salts and cyclic dipeptides). Multiple studies suggest that these compounds affect the activation state of ToxR by interacting with its PPD (10, 12, 19, 23). This is evidenced by recent studies showing that bile salts directly bind to the PPD (19). This binding destabilizes the PPD and promotes heterodimer formation with ToxS to activate ToxR. We suspect that indole may function by a similar mechanism to activate ToxR. Indole has been suggested to be a protein folding inhibitor (55, 56). This was documented for AsqR, a LuxR-type regulator, where indole blocked AsqR folding to inhibit its interaction with quorum-sensing molecules (56). In the case of ToxR, if indole blocked PPD folding, it would presumably promote interaction of the PPD with ToxS and lead to the formation of an activated ToxR. The facts that ToxR-mediated expression of *leuO* in the presence of extracellular indole was dependent on the PPD (Fig. 4) and that the PPD was dispensable for OmpU and CT/TCP production (23, 57) are consistent with this hypothesis, but additional work is required to confirm this. Based on these results, we propose that indole is a ToxR agonist.

Deletion of *leuO* only partially restored CT production during growth in the presence of indole (Fig. 3). This suggested that indole also inhibited virulence factor production by a second LeuO-independent mechanism. Although we were unable to determine this mechanism, we note that virulence regulation is linked to central metabolism via ToxT. Diminished respiration and tricarboxylic acid (TCA) cycle activity were reported to increase *toxT* expression (58). ToxT was also reported to repress a number of genes involved in carbon metabolism (15), suggesting a feedback loop between central metabolism and ToxT. Since metabolism genes represented one of the largest categories of indole-regulated genes (Table 1), it is possible that indole-dependent effects on metabolism may have contributed to virulence repression. Among the indole-regulated metabolism genes were genes involved in the TCA cycle (*sdhCD*) and glycolysis (*gapA* and *lld*) that were previously reported to be regulated by ToxT (15). This suggests that indole may regulate *V. cholerae* virulence by altering cell metabolism. We previously reported that ToxR can function as a metabolic sensor (23). This conclusion was supported by the fact that the TCA cycle intermediate malate impacts ToxR activation. Malate production was also previously linked to *toxT* expression (58). Thus, we speculate that indole-mediated effects on metabolism may lead to the generation and accumulation of metabolites that inhibit virulence by directly impacting ToxT or through metabolite-dependent effects on ToxR activation.

The ability to form biofilms is critical to the *V. cholerae* life cycle. Biofilm-associated *V. cholerae* is present in rice-water stool (16), and biofilm formation has been reported to be required for the hyperinfectious phenotype observed in human- and animal-shed *V. cholerae* (31). Biofilm production genes were the most highly upregulated genes in cells grown in the presence of indole (Table S2). This finding corroborates a previous

study showing that indole promoted biofilm formation in environmental *V. cholerae* isolates (30). Here, we extended these studies by documenting that indole-mediated biofilm production was dependent upon ToxR and LeuO (Fig. 5). ToxR was shown to directly regulate the transcription of *vps* genes (19), whereas *leuO* deletion was reported to diminish biofilm production (14). Our results are consistent with these findings. Interestingly, we observed that deletion of the ToxR PPD diminished biofilm production. Given that ToxR PPD was previously shown to be dispensable for *ompU* expression and for induction of the ToxR virulence regulon (23, 57), this finding indicates that there are differential requirements for the PPD at specific ToxR target promoters. The observation that the ToxR PPD was required for indole-promoted biofilm production provides additional support for the conclusion that ToxR senses indole via its PPD.

In addition to promoting biofilm production, indole was reported to activate the expression of the type VI secretion system in environmental *V. cholerae* isolates (30). This is an intriguing result in light of the high concentrations of indole that are present in the human gut and recent studies showing the contribution of the T6SS to *V. cholerae* pathogenesis (59). Here, we were not able to confirm indole-dependent T6SS activation in the O1 El Tor strain N16961. We suspect that this may be due to strain-specific differences since most toxigenic strains do not exhibit T6SS activity *in vitro* (40). Thus, it is still possible that indole affects T6SS production *in vivo*. Additional work will be required to address this.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains, plasmids and oligonucleotides used in this study are listed in Table S1. *V. cholerae* O1 El Tor Δ*lacZ* strain N16961 (JB58) was used as the WT (60) in all experiments unless otherwise noted. *Escherichia coli* strains EC100λ*pir* and SM10λ*pir* were hosts for cloning experiments and conjugation, respectively. All bacterial strains were maintained at −80°C in 25% glycerol and grown in lysogeny broth or agar (61). *V. cholerae* was grown in AKI broth for induction of the ToxR regulon, as previously described (62). Minimal T medium was prepared as previously described (44). Amino acids (Fisher Scientific) were dissolved in water and used at final concentration of 12.5 mM total amino acids. Antibiotics were used in the following concentrations: streptomycin (Sm), 100 μg/ml; carbenicillin (Cb), 100 μg/ml; and kanamycin (Km), 25 μg/ml. A fresh indole (Acros Organics) stock solution was prepared in methanol on the day of each experiment; control cultures received an equivalent volume of methanol.

Mutant construction. The allelic-exchange vector pWM91::Δ*tnaA* was created by crossover PCR, as previously described (63). Briefly, VCA0161-F1/-R2 and VCA0161-F2/-R1 PCR primer pairs were used in separate reactions with N16961 genomic DNA. The resulting ~1-kb PCR amplicons were pooled and then used as the template for a second PCR using flanking VCA0161-F1/-R1 PCR primers. The resulting ~2-kb amplicon was restricted with SacI and BamHI endonucleases before being ligated with similarly digested pWM91 to generate pWM91::Δ*tnaA*. Deletion of *tnaA* was accomplished by conjugating pWM91::Δ*tnaA* into *V. cholerae* strain JB58 and selecting cointegrants for Cb/Sm resistance. Sm- and Cb-resistant cointegrants were then plated on no-salt LB agar plates containing 5% sucrose, and sucrose-resistant and Cb-sensitive colonies were then screened by PCR using the VCA0161-F1/-R1 primers to confirm *tnaA* deletion.

RNA sequencing. *V. cholerae* strain JB58 was grown under AKI conditions with the addition of 0.5 mM indole or an equivalent amount of methanol for 3.5 h when the total RNA was isolated using TRIzol according to the manufacturer's directions (Invitrogen) and further purified using a RNeasy kit with in column DNase treatment (Qiagen). The resulting total RNA was then processed and sequenced by the University of Pittsburgh Health Sciences Sequencing Core at Children's Hospital of Pittsburgh. Briefly, the RNA samples were quantified using a Qubit 2.0 fluorimeter (Thermo Scientific), and the RNA integrity was assessed using an Agilent TapeStation 2220 system. The Ribo-Zero rRNA removal kit (Illumina) was used to deplete rRNA, and sequencing libraries were prepared and barcoded using a TruSeq Stranded Total RNA Library Prep kit (Illumina). The resulting libraries were pooled, and single-end sequencing was performed on an Illumina NextSeq 500 using a High-Output 75 cycle kit. The resulting FASTQ files from three independent experiments were mapped to the N16961 reference genome (61) using CLC Genomics Workbench (v10.1; Qiagen) and the default mapping parameters (i.e., mismatch cost, 2; insertion cost, 3; deletion cost, 3; length fraction, 0.8; similarity fraction, 0.8; strand specificity, both; maximum hits for a read, 10; expression value, total counts). Sample normalization (via the TMM method [64]) and identification of differentially expressed genes were accomplished using the Differential Expression for RNA-Seq tool in CLC Genomics Workbench. Genes showing a ≥1.5-fold difference in expression and a false discovery rate *P* value of ≤0.05 were defined as differentially expressed genes.

Quantification of indole production in *V. cholerae*. WT strain JB58 and an isogenic Δ*tnaA* mutant were inoculated 1:100 into LB broth, followed by incubation with shaking at 37°C. Culture aliquots were then collected at the indicated times and subjected to centrifugation to separate the cells from the culture supernatant. Aliquots of the supernatants were then used to quantify indole production using a

hydroxylamine-based indole assay, as previously described (65). Briefly, freshly prepared indole standards ranging from 0 to 1 mM were prepared in methanol. In a microtiter plate, 100-μl portions of the indole standards and *V. cholerae* culture supernatants were added in triplicate, followed by incubation for 15 min at room temperature with 5.3 M NaOH and 0.3 M hydroxylamine hydrochloride (NH_2OH-HCl). After incubation, 125 μl of 2.7 M sulfuric acid (H_2SO_4) was added to each well, and the plates were incubated at room temperature for up to 30 min before indole production was measured spectrophotometrically in a BioTek XL808 plate reader at 530 nm. The concentration of indole in *V. cholerae* culture supernatants was then determined relative to the standard curve.

Gene reporter assays. β-Galactosidase assays were performed as follows. Overnight cultures of *V. cholerae* strain JB58 carrying the indicated *lacZ* promoter reporters were diluted 1:5,000 into AKI medium containing 0.5 mM indole or an equivalent amount of methanol and incubated at 37°C under AKI growth conditions. Culture aliquots were then collected in triplicate at 5 h to quantify β-galactosidase production, as previously described (66). Gene expression assays done in minimal T medium were performed as follows: 1-ml portions of overnight cultures of the indicated *V. cholerae* strains carrying *toxRS-lacZ*, *ompU-lacZ*, or *leuO-lacZ* reporters were pelleted and washed twice in T medium before being subcultured at a 1:100 dilution into fresh T medium containing 0.5 mM indole or an equivalent amount of methanol at 37°C with shaking. Culture aliquots were collected in triplicate at 4 h (optical density at 600 nm [OD_{600}] ~0.3) to quantify the β-galactosidase activity.

Indole agonist activity. An OmpU/OmpT porin-switching assay was used to assess whether indole was a ToxR agonist (44). ToxR directly regulates *ompU* and *ompT* expression, activating *ompU* and repressing *ompT* (42, 43). In rich medium (and *in vivo*) OmpU is predominantly produced. In contrast, OmpT predominates in minimal medium. However, the addition of specific additives to minimal media causes a porin switch that results in increased OmpU production. This porin switch is mediated by two independent mechanisms: (i) increased *toxR* expression or (ii) ToxR protein activation (44). The addition of an amino acid cocktail of asparagine, arginine, glutamate, and serine (NRES) to minimal medium results in porin switching due to increased *toxR* transcription. The concomitant increase in ToxR protein is enough to activate *ompU* transcription and repress *ompT* transcription. The second mechanism is agonist dependent, whereby adding ToxR agonists to minimal medium activates preformed ToxR proteins (i.e., it does not affect *toxR* expression or ToxR abundance). This has been demonstrated with bile salts and cFP, which are thought to interact with the ToxR PPD to facilitate the formation of an activated conformation (10, 12, 19).

ToxR and OmpU immunoblotting. *V. cholerae* strain JB58 and an isogenic Δ*toxRS* mutant were grown overnight in minimal T medium. The overnight cultures were then diluted 1:100 into fresh T medium containing the amino acid cocktail NRES (12.5 mM total amino acids), 0.5 or 1 mM indole, or an equivalent amount of methanol. The resulting cultures were then grown for 4 h (OD_{600} of ~0.5) before aliquots were collected and normalized by cell density. Equal volumes from each sample were then suspended in Laemmli solubilization buffer, followed by heating at 100°C for 10 min before being resolved on an SDS–10% PAGE gel. The production of ToxR and OmpU was determined by Western immunoblotting with antisera against ToxR or OmpU, respectively. Proteins on Western blots were visualized using the SuperSignal West Pico chemiluminescent detection kit (Pierce Biotechnology).

CT and TCP quantification. *V. cholerae* strain JB58 and an isogenic Δ*leuO* mutant were grown with or without the indicated concentrations of indole for 16 h under AKI conditions, after which culture aliquots were collected for quantification of CT and TCP. CT production was quantified by a GM1 enzyme-linked immunosorbent assay (ELISA) as previously described using purified CT (Sigma) as a standard (21). TCP production was determined by Western immunoblotting as previously described using polyclonal antisera against TcpA, the pilin subunit of TCP (21). Proteins on Western blots were visualized using a SuperSignal West Pico chemiluminescent detection kit (Pierce Biotechnology).

Biofilm assays. Biofilm formation was assessed by inoculating the indicated *V. cholerae* strains at a 1:1,000 ratio into 1 ml of LB medium containing 0 or 0.5 mM indole in 13-by-100-mm borosilicate glass tubes. Triplicate cultures for each test strain and test condition were then incubated at room temperature without shaking for 48 h. Biofilm formation was then visualized by decanting the culture media and staining the glass-adherent biofilm with 1% crystal violent solution. The test tubes were then washed three times with distilled water before biofilm production was quantified by destaining the crystal violet-stained biofilms with 5% acetic acid and measuring the absorbance of the resulting solution at 570 nm.

RNA sequencing data. The RNA-seq data have been deposited with links to BioProject accession number PRJNA374569 in the NCBI BioProject database.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00776-18.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.1 MB.

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