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Enhanced Interaction of *Vibrio cholerae* Virulence Regulators TcpP and ToxR under Oxygen-Limiting Conditions

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***Vibrio cholerae* is the causative agent of the diarrheal disease cholera. The ability of *V. cholerae* to colonize and cause disease requires the intricately regulated expression of a number of virulence factors during infection. One of the signals sensed by *V. cholerae* is the presence of oxygen-limiting conditions in the gut. It has been shown that the virulence activator AphB plays a key role in sensing low oxygen concentrations and inducing the transcription of another key virulence activator, TcpP. In this study, we used a bacterial two-hybrid system to further examine the effect of oxygen on different virulence regulators. We found that anoxic conditions enhanced the interaction between TcpP and ToxR, identified as the first positive regulator of *V. cholerae* virulence genes. We further demonstrated that the TcpP-ToxR interaction was dependent on the primary periplasmic protein disulfide formation enzyme DsbA and cysteine residues in the periplasmic domains of both ToxR and TcpP. Furthermore, we showed that in *V. cholerae*, an interaction between TcpP and ToxR is important for virulence gene induction. Under anaerobic growth conditions, we detected ToxR-TcpP heterodimers, which were abolished in the presence of the reducing agent dithiothreitol. Our results suggest that *V. cholerae* may sense intestinal anoxic signals by multiple components to activate virulence.**

The Gram-negative bacterium *Vibrio cholerae* is responsible for the diarrheal disease cholera. It is a facultative pathogen that resides predominantly in a variety of aqueous environments (1), and human infection normally starts with the ingestion of food or water contaminated with *V. cholerae*. Subsequently, the bacteria enter the small intestine, where they must then penetrate the mucus lining to colonize the epithelial cells beneath and cause disease (2). As it colonizes, *V. cholerae* produces an array of virulence factors, including cholera toxin (CT) and toxin-coregulated pili (TCP) (3, 4). These virulence factors are transcriptionally regulated by multiple systems (5). The primary, direct transcriptional activator of the *V. cholerae* genes encoding CT and TCP is ToxT (6). Transcription of *toxT* is regulated, in turn, by the ToxR and TcpP proteins (7). The expression of *tcpP* is activated by two additional activators, AphA and AphB (8, 9), whereas the expression of *toxR* is thought to be constitutive and can be modulated by AphB (10). Both TcpP and ToxR are inner membrane proteins with N-terminal cytoplasmic domains homologous to winged helix-turn-helix transcriptional activators and C-terminal periplasmic domains (11). ToxR and TcpP can interact with each other (12, 13), but the exact mechanism by which ToxR facilitates TcpP-mediated activation of the *toxT* promoter is unclear. It has been hypothesized that ToxR binds to three helical turns upstream of TcpP in the *toxT* promoter (14) and recruits TcpP proteins to activate *toxT* transcription (13).

The environmental cues influencing the expression of virulence genes *in vivo* are not well characterized. It has been reported that certain environmental conditions such as temperature, osmolarity, pH, and iron availability influence the expression of virulence genes *in vitro* (15). For example, quorum-sensing systems negatively regulate virulence genes by repressing *aphA* expression through HapR (16–18). Fatty acids in bile and bicarbonate regulate ToxT activity (19, 20). We recently reported that a set of bile salts induce virulence genes by enhancing TcpP protein activity, possibly by promoting TcpP-TcpP intermolecular disulfide bond formation (21). Moreover, we found that under oxygen-limiting conditions in the gastrointestinal tract (22), *V. cholerae* virulence genes are highly expressed. We showed that oxygen-limiting conditions enhance the dimerization and activity of AphB, which leads to the production of virulence factors (23). To further examine whether, in addition to AphB, the activity of other virulence regulators may be affected by the oxygen concentration, we used a bacterial two-hybrid system to examine protein-protein interactions between subunits of virulence regulators. We found that while interactions between TcpP and TcpP, ToxR and ToxR, and ToxT and ToxT were insensitive to the environmental oxygen concentration, the interaction between ToxR and TcpP was increased under oxygen-limiting conditions. We further demonstrated that the periplasmic domains of both TcpP and ToxR are important for this interaction and cysteine residues in the periplasmic domains of both proteins were critical for the TcpP-ToxR interaction. We show that the anaerobiosis-enhanced TcpP-ToxR interaction contributes to *V. cholerae* virulence gene expression.

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MATERIALS AND METHODS

Strains, plasmids, and culture conditions. For the strains, plasmids, and primers used in this study, see Tables S1 to S3 in the supplemental material. All of the *V. cholerae* strains used in this study were derived from E1 Tor C6706 (24) and propagated in Luria broth (LB) containing appropriate antibiotics at 37°C. Cultures were grown aerobically (with shaking at 250 rpm), microaerophilically (stationary growth), or anaerobically (Vinyl Anaerobic Chambers; Coy Laboratory Products) unless otherwise noted. *Escherichia coli* strains DH5α and DH5α λpir were used for cloning. *E. coli* XL1-Blue was used for the bacterial adenylate cyclase two-hybrid (BACTH) system (25) and recombinant fusion construct propagation. *E. coli* BTH101 (Δ*cyaA*) (25) was used for bacterial hybrid analysis. Full-length *toxR* or *tcpP*, truncated fragments thereof, and cysteine mutant derivatives were PCR amplified and cloned into the inducible expression vectors pUT18C and pKT25 (25), respectively. The arabinose-inducible P_{BAD}-*tcpPH* plasmids were described in reference 21. The isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible P_{lac}-*toxRS* plasmid was constructed by PCR amplifying full-length *toxRS* genes and cloning them into the pSRKtc vector (26). The P_{ctxA}-*luxCDABE* transcriptional reporter plasmid was constructed by cloning the promoter of *ctxA* sequences into the pBBR-*lux* plasmid (27). The *tcpA-sh ble* construct was described previously (28). Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; streptomycin, 100 μg/ml; kanamycin, 50 μg/ml. For *E. coli*, we used the following: chloramphenicol, 30 μg/ml; tetracycline, 10 μg/ml. For *V. cholerae*, we used the following: chloramphenicol, 2 μg/ml; tetracycline, 2 μg/ml, streptomycin, 100 μg/ml.

Bacterial two-hybrid system for analysis of the ToxR-TcpP interaction. Overnight cultures of *E. coli* BTH101 or BTH101Δ*dsbA* containing plasmid pKT25-ToxR (and its derivatives) and pUT-18C-TcpP (and its derivatives) were subcultured in LB containing 0.5 mM PTG and grown aerobically (with shaking at 250 rpm), microaerophilically (stationary growth), or anaerobically (anaerobic incubator) at 37°C until the optical density at 600 nm (OD_{600}) reached approximately 0.3. β-Galactosidase activities were then measured as described previously (29). The *toxR* constructs contain full-length *toxS* in the same operon with *toxR*, and the *tcpP* constructs contain full-length *tcpH* in the same operon with *tcpP*.

Measurement of *toxT* transcription in *V. cholerae*. The P_{toxT}-*lacZ* plasmid reporter was constructed by cloning *toxT* promoter DNA into LacZ transcriptional reporter plasmid pAH6 (30). Overnight cultures of the *V. cholerae* Δ*tcpPH* Δ*toxRS* mutant containing P_{toxT}-*lacZ* reporters and various P_{BAD}-*tcpPH* and P_{tac}-*toxRS* plasmids were diluted 1:50 (for microaerophilic growth) or 1:2,000 (for aerobic growth) into fresh AKI medium (1.5% Bacto peptone, 0.4% yeast extract, 0.5% sodium chloride, pH 7.5) supplemented with the appropriate antibiotics, 0.01% arabinose, and 1 μM IPTG and incubated at 37°C (stationary or shaking) until the OD_{600} reached 0.3. β-Galactosidase activities were then assayed as described previously (29).

Western blot assays and SDS-PAGE. Overnight cultures of wild-type or Δ*toxRS* Δ*tcpPH* mutant *V. cholerae* containing plasmids P_{BAD}-*tcpPH* and P_{lac}-*toxRS* were diluted into fresh AKI medium supplemented with appropriate antibiotics and the various concentrations of arabinose and IPTG indicated. The cultures were incubated at 37°C (stationary or shaking) until the OD_{600} reached 0.3. The cultures were then pelleted and resuspended in phosphate-buffered saline. Cell pellets normalized by the total protein amounts were then resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sampling buffer with or without dithiothreitol (DTT) and loaded at the same volumes for each sample onto a 15% polyacrylamide gel. Proteins were transferred to Immobilon-P SQ polyvinylidene difluoride transfer membrane for Western blot analysis with polyclonal rabbit anti-ToxR or anti-TcpP antibodies (10, 21), followed by enhanced-chemiluminescence anti-rabbit IgG secondary antibody (GE Healthcare). Proteins were detected by use of Thermo Scientific SuperSignal West Pico Chemiluminescent Substrate.

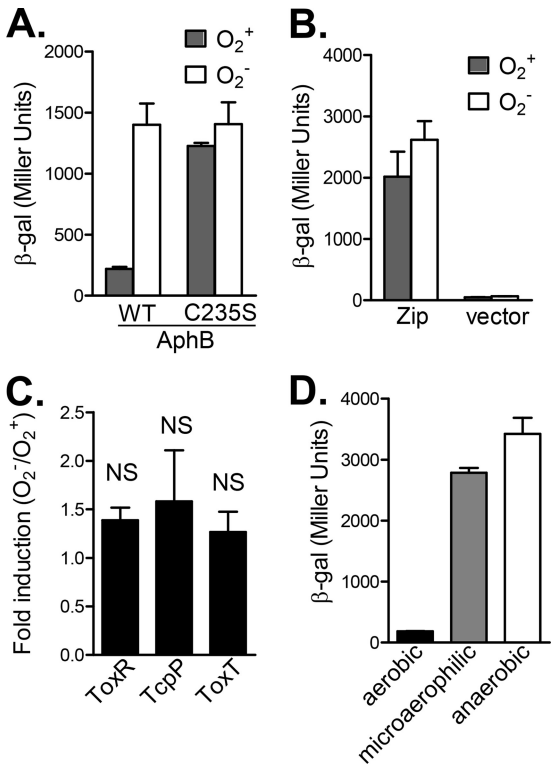

FIG 1 Effects of oxygen on protein interactions between *V. cholerae* virulence regulators in a bacterial two-hybrid system. The *E. coli* cAMP-based two-hybrid system was used in this study (25). See Materials and Methods for details. The BTH101 strains containing different combinations of T25-T18 fusion pairs were grown aerobically, microaerophilically, and anaerobically at 37°C until mid-log phase, and β-galactosidase activity was measured and recorded in Miller units (29). (A) Effects of oxygen on AphB-AphB and $AphB^{C235S}$-$AphB^{C235S}$ interactions. (B) Effects of oxygen on positive controls (leucine zipper of GCN4) and negative controls (empty vectors). (C) ToxR-ToxR, TcpP-TcpP, and ToxT-ToxT interactions. The ratios of β-galactosidase units from cultures grown anaerobically and aerobically are presented. (D) ToxR-TcpP interactions. The results are the mean of three experiments ± the standard deviation. β-gal, β-galactosidase; WT, wild type; NS, not significantly different.

RESULTS AND DISCUSSION

Effect of oxygen concentration on protein-protein interactions of *V. cholerae* virulence regulators. Protein-protein interactions have been shown to play critical roles in gene regulation in all living organisms. There are a number of genetic approaches to the analysis of protein-protein interactions. Among them, a BACTH system is a proven, simple, and fast approach to the characterization of these interactions in *E. coli* cells (25). This system has been used to examine *V. cholerae* TcpP-ToxR (12) and TcpP-TcpP interactions in response to bile salt signals (21). To test whether this method is suitable for elucidation of the effects of oxygen on protein-protein interactions, we first constructed fusions of T25 and T18, both of which are complementary fragments of the catalytic domain of adenylate cyclase (CyaA) from *Bordetella pertussis* (25), with wild-type AphB and with a cysteine 235-to-serine AphB mutant that is insensitive to oxygen (23). We then measured the β-galactosidase activity in an *E. coli* Δ*cya* mutant strain grown aerobically or anaerobically. The AphB-AphB interaction brings the two Cya fragments together and leads to cyclic AMP (cAMP) generation and increased β-galactosidase production. Figure 1A

shows that wild-type AphB proteins interacted significantly better under anaerobic growth than under aerobic growth, whereas the strength of interaction between AphB^{C235S} mutant proteins was similar, regardless of the oxygen conditions during growth. These results are consistent with our previous findings (23) that oxygen-limiting conditions enhance AphB dimerization and AphB^{C235S} mutant proteins form dimers even during aerobic growth. In addition, oxygen availability did not affect β-galactosidase production in the strains containing either leucine zipper of GCN4 T18/25 (25) fusions (positive controls) or empty vectors (negative controls) (Fig. 1B). These data suggest that the BACTH system is suitable for assaying the effects of growth conditions on protein-protein interactions. We then used this system to examine whether interactions of other *V. cholerae* virulence regulators such as ToxR, TcpP, and ToxT are affected by the oxygen concentration. We found that ToxR proteins could interact with each other to activate β-galactosidase production, but this induction was not oxygen concentration dependent (Fig. 1C). Similarly, TcpP-TcpP and ToxT-ToxT interactions were also O_2 independent (Fig. 1C, second and third columns). Of note, we always included *tcpH* with *tcpP* and *toxS* with *toxR* on the T18/T25 fusion plasmids to avoid TcpP and ToxR protein instability (31, 32). Finally, we examined TcpP-ToxR interaction when bacteria were grown aerobically, microaerophilically, or anaerobically. Figure 1D shows that under oxygen-limiting conditions during either microaerophilic or anaerobic growth, the TcpP-ToxR interaction was greatly enhanced, suggesting that, in addition to modulating AphB dimerization, anaerobiosis also influences other important components of the virulence regulatory cascade.

Periplasmic domains of ToxR and TcpP are critical for the anaerobiosis-dependent TcpP-ToxR interaction. To investigate the mechanism of an anaerobiosis-enhanced ToxR-TcpP interaction, we first examined how truncated TcpP proteins affect this interaction. We introduced T18 fusions of full-length TcpP or TcpP truncations into *E. coli cyaA* mutants containing the T25 fusion of full-length ToxR and assayed for LacZ production during aerobic or anaerobic growth. Figure 2A shows that compared to that of wild-type TcpP, truncated TcpP containing only the cytoplasmic domain or the cytoplasmic and transmembrane (TM) domains could not interact with ToxR under anaerobic conditions. Truncated TcpP containing both the periplasmic and TM domains, as well as chimeric proteins consisting of the TcpP periplasmic domain and the TM domain of ToxR-TcpP and the ToxR cytoplasmic domain, however, could partially interact with ToxR in the absence of oxygen. We examined the levels and localization of the proteins produced by those constructs in *E. coli* and found that they produced similar amount of proteins and that they were localized at the predicted locations (data not shown; 21). These data suggest that the periplasmic domain of TcpP is critical for interaction with ToxR under oxygen-limiting conditions.

To examine the domain requirement of ToxR, we tested the interaction between a T18 fusion of full-length or truncated ToxR and a T25 fusion of full-length TcpP by assaying β-galactosidase production under aerobic or anaerobic conditions. We found that ToxR's periplasmic domain was also important for interaction with TcpP under anaerobic conditions, as LacZ activity was abolished in any constructs where the periplasmic domain of ToxR was deleted (Fig. 2B). These data indicate that the ToxR periplasmic domain contributes to the anaerobiosis-enhanced interaction

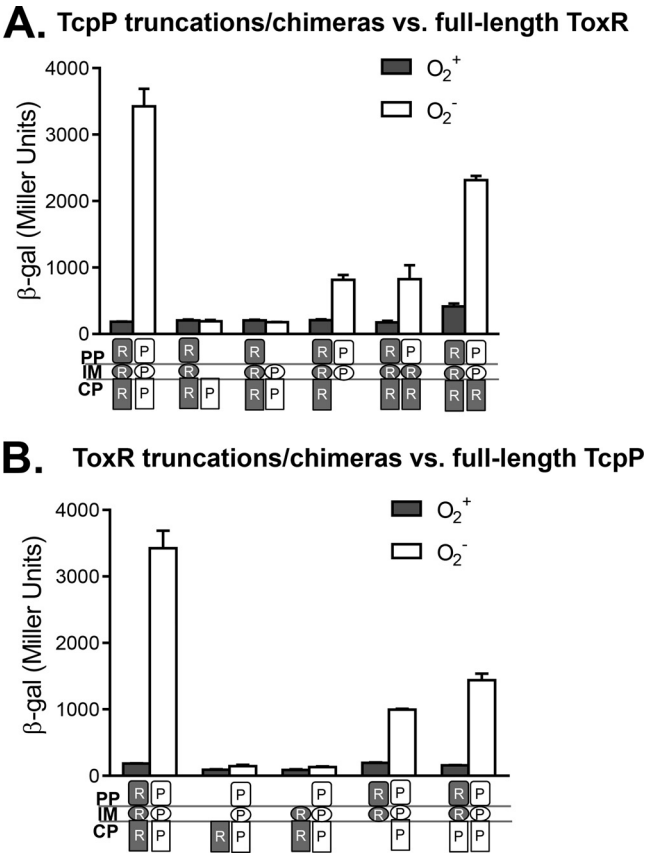

FIG 2 Domain analysis of effects of oxygen on ToxR-TcpP interactions. (A) Full-length ToxR interacts with various TcpP truncations and chimeras. (B) Full-length TcpP interacts with various ToxR truncations and chimeras. The BTH101 strains containing those pairs were grown aerobically (gray bars) or anaerobically (white bars) at 37°C to an OD_{600} of ~0.3, and β-galactosidase activity was measured and recorded in Miller units (29). β-gal, β-galactosidase; PP, periplasm; IM, inner membrane; CP, cytoplasm. White symbols, TcpP (P) domains; shaded symbols, ToxR (R) domains.

with TcpP proteins, likely through the TcpP periplasmic domain. Intriguingly, Crawford et al. previously showed that the periplasmic domain of ToxR is not required for TcpP-dependent activation of virulence (33). In that study, a classical strain of *V. cholerae* was used and the cultures were grown aerobically at 30°C. It will be interesting to examine whether virulence genes are further induced under anaerobic conditions in classical strains.

Disulfide bond formation is required for the ToxR-TcpP interaction. It has been shown that intermolecular disulfide bond formation plays an important role in bile salt-induced TcpP activity (21). To examine whether disulfide bond formation is involved in the ToxR-TcpP interaction, we first tested the bacterial two-hybrid system in a *dsbA* mutant, which lacks the ability to form disulfide bonds in periplasmic proteins (21). DsbA is a primary periplasmic protein thiol:disulfide oxidoreductase found in eukaryotic endoplasmic reticulum and in the bacterial periplasm. It oxidizes about 300 proteins exported to the periplasm in *E. coli* (34, 35). We found that compared to that of the wild type, the ToxR-TcpP interaction was significantly reduced in the *dsbA* mutant. The positive-control plasmids that express Zip-T18/T25 fusions still produced similar amount of LacZ in both the *dsbA*$^+$ and *dsbA* mutant backgrounds (data not shown), indicating that the

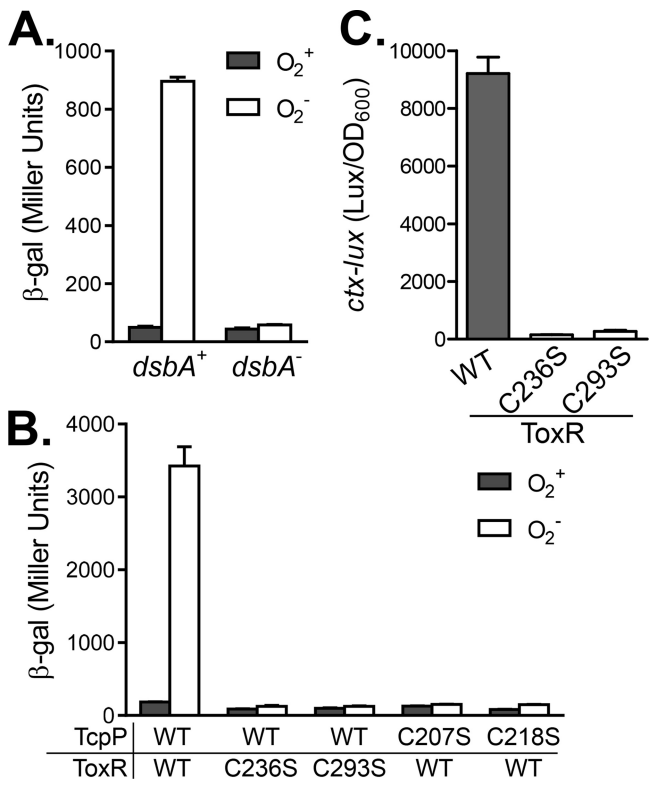

FIG 3 Effects of periplasmic disulfide bonds and cysteine residues on a ToxR-TcpP interaction. (A) ToxR-TcpP interaction in the *dsbA* mutant. BTH101 and BTH101 Δ*dsbA* containing pKT25-ToxRS and pUT18C-TcpPH were grown aerobically and anaerobically at 37°C to an OD_{600} of ~0.3. β-Galactosidase (β-gal) activity was then measured and recorded in Miller units. (B) Effects of periplasmic cysteine mutations. BTH101 containing different combinations of ToxR and TcpP cysteine mutant proteins were grown aerobically and anaerobically at 37°C to an OD_{600} of ~0.3. β-Galactosidase activity was then measured. (C) Effect of ToxR cysteine mutations on *ctx* expression in *E. coli*. *E. coli* strains containing P_{ctxA}-*luxCDABE* reporter and P_{lac}-*toxR* (wild type and its cysteine mutant derivatives) plasmids were grown in LB containing 0.5 mM IPTG to an OD_{600} of ~0.3. Luminescence was then measured and reported as light units/OD_{600} unit. Data represent the mean ± the standard deviation of three independent experiments. WT, wild type.

two-hybrid system, in general, is functional in *dsbA* mutants. There are two cysteine residues in the periplasmic region of ToxR (C236 and C293) and TcpP (C207 and C218). To test which cysteine residues are involved in the ToxR-TcpP interaction, we mutated each cysteine residue to a serine in the periplasmic domains of ToxR and TcpP and examined the effects of these changes on the ToxR-TcpP interaction with or without oxygen. Figure 3B shows that mutation of either C236 or C293 of ToxR abolished the ToxR-TcpP interaction independently of the oxygen concentration. Similarly, cysteine mutant TcpP also failed to interact with ToxR. These data suggest that these cysteine residues are critical for the ToxR-TcpP interaction. However, we could not rule out the possibility that mutations in cysteine residues may change the protein's conformation and therefore cause loss of stability. Previously, we showed that the C207S mutant of TcpP is inactive whereas the C218S mutant is active even in the absence of bile salts (21). The ToxR periplasmic domain has been proposed to act as a sensor of environmental stimuli and contains two cysteine residues that can form both intra- and intermolecular disulfide bonds (36). Interestingly, Fengler et al. have recently reported that the double cysteine-to-serine mutation of ToxR does not affect virulence gene activation but does abolish the ability of ToxR to activate *ompU* (37). The reason for this phenotype remains unclear. To examine whether ToxR periplasmic cysteine residues are important for its function in our systems, we compared the abilities of wild-type ToxR and single cysteine mutant ToxR to activate *ctx* expression in *E. coli*. We found that both cysteine residues in ToxR were important for ToxR activity, as both single cysteine mutations virtually abolished ToxR activity in *E. coli* (Fig. 3C). We do not know why our data are inconsistent with the previous report (37), and we cannot rule out the possibility that either ToxR or TcpP cysteine mutations interrupted interactions by changing their conformation, rather than the involvement of disulfide bond formation. Moreover, it has been reported that in the absence of ToxS, ToxR interchain disulfide bond formation is enhanced (37). It will be interesting to examine the TcpP-ToxR interaction in the absence of ToxS in the future.

Oxygen limitation-dependent interaction of TcpP and ToxR is important for virulence induction in *V. cholerae*. We have shown that the ToxR-TcpP interaction was enhanced under oxygen-limiting conditions and that this interaction was dependent on DsbA and cysteine residues in both ToxR and TcpP periplasmic domains. To investigate whether this interaction is also important for virulence activation in *V. cholerae*, we first decoupled the expression of *tcpP* from the oxygen concentration. It has been reported that *tcpP* expression was increased under oxygen-limiting conditions because of the thiol-based switch of AphB (23). We developed a controllable system to express *tcpPH* and *toxRS* independently of oxygen by placing their coding sequences under the control of the P_{BAD} and P_{lac} promoters, respectively. We introduced these two plasmids into a mutant strain of *V. cholerae* lacking both *toxRS* and *tcpPH*. We then performed Western blot analysis to determine the amounts of arabinose and IPTG inducers needed to express amounts of TcpP and ToxR similar to those of the wild type under anaerobic condition. We found that using 0.01% arabinose and 1 μM IPTG to induce *tcpP* and *toxR*, respectively, could achieve wild-type levels of production of these proteins (Fig. 4A). Therefore, we used these conditions to express *tcpPH* and *toxRS* in the Δ*toxRS* Δ*tcpPH* mutant to examine the effects of oxygen on the TcpP-ToxR interaction by measuring *toxT* expression. We found that both ToxR and TcpP are required for *toxT* expression, as the addition of either arabinose or IPTG failed to induce *toxT* expression (see Fig. S1 in the supplemental material). With both inducers, this strain showed strong *toxT* expression when cultures were grown anaerobically but not when they were grown aerobically (Fig. 4B). Similarly, the expression of *tcpA*, the major virulence determinant in the same strain background and under the same inducing conditions was strongly enhanced under anaerobic conditions (see Fig. S2 in the supplemental material). Taken together, these data suggest that anaerobically enhanced TcpP-ToxR interaction contributes to virulence induction. In the *dsbA* mutant of *V. cholerae*, *toxT* was not expressed (Fig. 4B), suggesting that disulfide bond formation is involved in the ToxR-TcpP interaction in *V. cholerae*. Furthermore, we examined the role of cysteine residues in periplasmic domains of ToxR and TcpP in *toxT* activation. Figure 4C shows that *toxT* expression was low when the $TcpP^{C207S}$, $ToxR^{C236S}$, and $ToxR^{C293S}$ mutant proteins were present; however, a $TcpP^{C218S}$ mutant protein could induce *toxT* expression even under aerobic

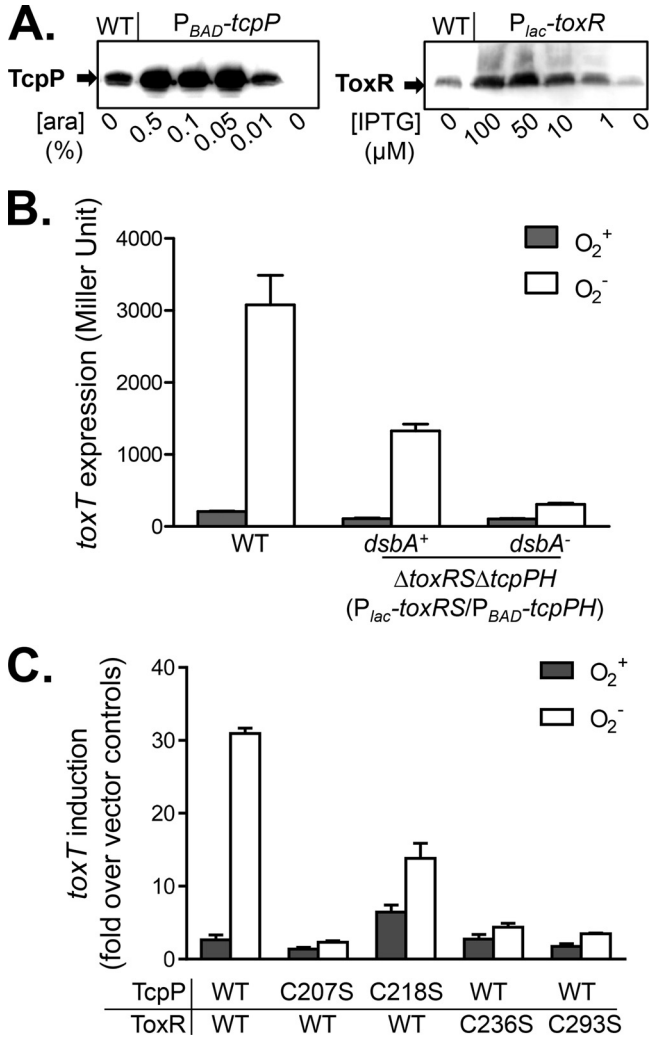

FIG 4 Effects of oxygen on the ToxR-TcpP interaction and virulence expression in *V. cholerae*. (A) Concentrations of inducers needed to express TcpP and ToxR to wild-type (WT) levels. Cultures of wild-type or Δ*toxRS* Δ*tcpPH* mutant *V. cholerae* containing P_{BAD}-*tcpPH* and P_{lac}-*toxRS* were grown in AKI medium in the presence of various amounts of arabinose (ara) or IPTG without shaking at 37°C to an OD_{600} of ~0.3. The cell pellets were subjected to Western blot assays with anti-TcpP (left panel) and anti-ToxR (right panel) antibodies. (B) Analysis of ToxR-TcpP interactions and activation of *toxT*. Wild-type *V. cholerae* (P_{toxT}-*lacZ*), a Δ*toxRS* Δ*tcpPH* mutant, and a Δ*toxRS* Δ*tcpPH* Δ*dsbA* mutant containing P_{BAD}-*tcpPH*, P_{lac}-*toxRS*, and P_{toxT}-*lacZ* reporter plasmids were grown aerobically or anaerobically in AKI medium supplemented with 0.01% arabinose and 1 μM IPTG to an OD_{600} of ~0.3. β-Galactosidase activity was then measured. Data represent the mean ± the standard deviation of three independent experiments. (C) Analysis of the effects of periplasmic cysteine mutations on ToxR-TcpP interactions and activation of *toxT*. The Δ*toxRS* Δ*tcpP* mutant (21) containing a P_{BAD}-*tcpPH* (or its cysteine→serine derivatives), P_{lac}-*toxRS* (or its cysteine→serine derivative mutant forms), or P_{toxT}-*lacZ* reporter plasmid was grown aerobically or anaerobically in AKI medium supplemented with 0.01% arabinose and 1 μM IPTG to an OD_{600} of ~0.3. β-Galactosidase activity was then measured. Data represent the mean ± the standard deviation of three independent experiments.

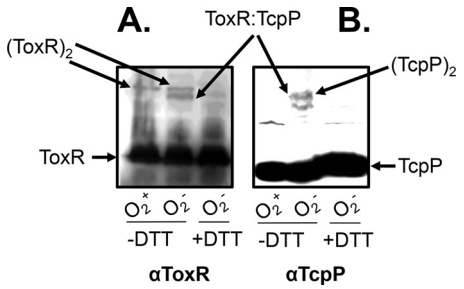

FIG 5 ToxR-TcpP interactions in *V. cholerae*. The Δ*toxRS* Δ*tcpPH* mutant harboring P_{lac}-*toxRS* or P_{BAD}-*tcpPH* was grown aerobically or anaerobically in AKI medium at 37°C and induced with 0.01% arabinose and 1 μM IPTG. Cell pellets were collected and resuspended in SDS-PAGE sampling buffer with or without DTT. Samples containing 0.1 μg total proteins were loaded onto a 15% polyacrylamide gel and subjected to Western blot analysis with anti-ToxR antibody (A) or anti-TcpP antibody (B).

conditions. Previously, we have shown that a $TcpP^{C218S}$ mutant protein could activate virulence in the absence of virulence-inducing bile salts because of the intermolecular disulfide bond formation between the mutant proteins (21). This may explain why this mutant protein could activate *toxT* in the presence of oxygen. More importantly, under anaerobic conditions, *toxT* induction by the $TcpP^{C218S}$ mutant protein was lower than that by wild-type TcpP. As $TcpP^{C218S}$ could not interact with ToxR (Fig. 3B), these results again suggest that TcpP-ToxR interactions under anaerobic conditions may be important for virulence activation.

To further demonstrate that TcpP-ToxR interacts better under anaerobic conditions, we performed a Western blot analysis with cultures of the *V. cholerae* Δ*toxRS* Δ*tcpPH* (P_{BAD}-*tcpPH*) (P_{lac}-*toxRS*) mutant strain grown in the presence of 0.01% arabinose and 1 μM IPTG aerobically and anaerobically. We used anti-ToxR (Fig. 5A) and anti-TcpP (Fig. 5B) antibodies. We found that in the absence of the reducing agent DTT, a band corresponding to a ToxR dimer (65 kDa) was present in the sample obtained from the aerobically grown culture, whereas an additional 58-kDa band that corresponds to the size of a ToxR-TcpP heterodimer was present in the sample from the anaerobically grown culture (Fig. 5A). This 58-kDa band was absent from the anaerobically grown culture when no TcpP was expressed (data not shown) and from the sample treated with DTT (Fig. 5, the last lane). Similarly, ToxR-TcpP heterodimers were detected only in anaerobically grown cultures without DTT treatment when anti-TcpP antibody was used (Fig. 5B). These data suggest that the ToxR-TcpP interaction is enhanced during anaerobic growth and that this interaction may be covalently linked through disulfide bond formation.

V. cholerae depends on a complex transcriptional cascade to activate virulence and colonization genes to survive in the host. One of the environmental signals used by *V. cholerae* to regulate virulence is an anoxic signal (23, 38), as gastrointestinal tracts are generally considered to be oxygen deficient (39). Reduced forms of AphB have been shown to activate *tcpP* transcription under oxygen-limiting conditions (23). Here, we discovered that ToxR-TcpP interacted better under these conditions. This interaction also played an important role in activating virulence gene expression. We further demonstrated the importance of the cysteine residues in the periplasmic domains of ToxR and TcpP in ToxR-TcpP interactions. Intriguingly, we recently showed that a set of bile salts serve as virulence inducers by promoting TcpP intermolecular disulfide bond formation (21). Interestingly, the AKI medium used to culture *V. cholerae* in this study may contain sufficient bile salts. It has been reported that Bacto peptone, the major

component of AKI medium, has approximately 10 mg/g of bile salts (40). Further investigation is required to better understand the relationships among periplasmic disulfide bond formation, ToxR-TcpP interaction, and virulence induction in host intestinal environments.

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