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TcpP L152A Constitutively Activating Virulence Gene Expression in *Vibrio cholerae*

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Abstract

Vibrio cholerae, the causative agent of severe watery diarrheal disease cholera, requires production of a number of virulence factors during infection which results from the activity of a cascading system of regulatory factors by sensing to different environmental signals. TcpP, a membrane-localized transcription activator in *V. cholerae*, activates virulence factors production by responding to human host signals. To better characterize the transmembrane helix in regard to its roles on TcpP positive effectors sensitivity, site-directed mutagenesis was performed to identify specific mutations in this region which could enhance TcpP transcription activity in the absence of stimuli, like bile salts. We found that TcpP L152A constitutively forms homodimer and activates *toxT* expression in the absence of bile salts. However, being active, TcpP L152A needs to form disulfide bonds between the cysteine residues in the periplasmic domain of TcpP. We also found that TcpP L152A showed a competitive advantage in the infant mouse colonization model by coadministrating the bile salt-sequestering resin cholestyramine. All these results demonstrate that the transmembrane helix of TcpP plays an important role in regulating TcpP transcription activity in response to its positive effectors.

Introduction

Vibrio cholerae is an aquatic organism and causes the acute diarrheal disease cholera. The ecological niche of *V. cholerae* is in the aquatic environment and humans get infection by drinking contaminated water [1]. When *V. cholerae* enters into the human small intestine, a set of critical virulence determinants which are required for successful colonization and causing human disease, like cholera toxin (CT) and the toxin-coregulated pilus (TCP) are specifically activated to produce by the chemical signals present in the small intestine [2, 3]. The production of these virulence factors in *V. cholerae* is under the control of a complex cascade of regulatory events [3, 4]. By sensing the anoxic environment of the small intestine, AphB, together with AphA, activates transcription of the transmembrane transcription factor TcpP, which in turn activates toxT transcription together with ToxR [5, 6]. ToxT is the transcription regulator that directly activates the transcription of the *ctx, tcp*, and *acf* genes, as well as additional genes within the Vibrio Pathogenesis Island (VPI) [3, 7].

TcpP is a bitopic membrane-localized transcriptional activator containing the winged helix-turn-helix (W-HTH) DNA binding domain in its amino-terminal cytoplasmic domain which is responsible for the downstream gene promoter binding and transcription activating [8, 9]. However, the cytoplasmic domain of TcpP alone cannot activate *toxT* transcription but only the full-length of TcpP can [10, 11]. TcpP transmembrane domain containing one helix is followed by a periplasmic domain which was reported to be important for TcpP proteolysis, homodimerization, and interacting with other membrane-localized proteins, like ToxR and TcpH [10–13]. TcpP is associated with the distinct effector protein TcpH to form a proposed transcription activation complex TcpP/TcpH and activate downstream virulence gene expression in combination with another transcription activation complex ToxR/ToxS [9, 14]. The association of TcpP and TcpH in *V. cholerae* prevents TcpP from degradation by the regulated intramembrane proteolysis (RIP) [10, 15, 16]. We

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previously reported that the two cysteine residues C207 and C218 in the periplasmic domain are critical for TcpP forming homodimer and activating *toxT* transcription [11]. The bile salts taurocholate present in the small intestine induce TcpP forming intermolecular disulfide bonds between C207 and C218 indirectly mediated by DsbA [17]. Another chemical signal calcium ion present in the small intestine enhances TcpP transcription activity by altering protein movement in the inner membrane [18], which suggests that TcpP transmembrane helix may also play an important role on affecting its activity.

Therefore, the goals of this study were to better characterize this transmembrane helix in regard to its roles on TcpP positive effectors sensitivity. We performed site-directed alanine mutagenesis of TcpP amino acids 141 to 163 to identify specific amino acid changes that alter TcpP activating *toxT* transcription.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

Strains, plasmids, and oligonucleotides used in this study are summarized in Table 1. All *V. cholerae* strains used in this study were derived from E1 Tor C6706 [19] unless otherwise noted, and were propagated in Luria Bertani (LB) media containing appropriate antibiotics at 37 °C.

Transcriptional *lux* reporters of *toxT* promoter regions in the pBBR-lux vector [22] have been described previously [11]. TcpP alanine mutations were constructed by overlap extension PCR [23]. Plasmids for overexpressing TcpP or mutants were either described previously [11] or constructed by cloning the PCR-amplified coding regions into pBAD24. Chromosomal replacement of WT *tcpP* with alanine mutant was constructed as described in [11]. Briefly a fragment of P_{tcpP}-*tcpP* L152A was cloned into a suicide vector containing a *V. cholerae* intergenic fragment between VCA0104 and VCA0105. The resulting plasmids were introduced into *V. cholerae* a *tcpP* partial deletion mutant (TcpPΔ67–89), in which *tcpH* is still intact, by selecting for double-crossover recombination events.

Measurement of Virulence Gene Expression and Virulence Factor Production

TcpP transcription activity assays were performed as described previously [11]. Briefly *V. cholerae* strains containing a plasmid-borne WT or mutated TcpP proteins expressed under the control of P_{BAD} promoter and a P*toxT–luxCDABE* transcriptional fusion plasmid were grown in LB broth containing 0.01% arabinose at 37 °C in the presence and absence of 1 mM taurocholate until $OD_{600} \approx 0.2$. Luminescence was measured using a Bio-Tek Synergy HT spectrophotometer to determine the activation levels of *toxT–lux*. Luminescence was measured and reported as light units/OD_{600}.

TcpP–TcpP Interaction Assay by a Bacterial Two-Hybrid System

TcpP homodimerization was tested by a bacterial two-hybrid system. Full-length TcpP WT or L152A were fused with the T25 and T18 domains of adenylate cyclase (CyaA) from *Bordetella pertussis*, respectively, and the T25, T18 fusion pairs were introduced into *E. coli cyaA* mutants [21]. Cultures were grown at 30 °C in the presence (+) and absence (−) of 1 mM TC for 8 h and β-galactosidase activity was measured and reported as Miller Units [24].

In Vivo Competition

In vivo colonization competition assays in infant mice were carried out in strict accordance with the animal protocols that were approved by the Institutional Animal Care and Use Committee of Zhejiang A&F University (Permit Number: ZJAFU/IACUC_2011-10-25-02). 5-day-old ICR mice purchased from the animal facility of Zhejiang Academy of Medical Sciences were raised in the animal facility of Zhejiang A&F University and were separated from their dams 1 h before infection. Subsequently, they were anesthetized by inhalation of isoflurane gas and then inoculated by oral gavage with of an appropriate dilution of the 1:1 mixture (WT and mutant) in 50 μL of PBS or PBS containing 1% (wt/vol) cholestyramine, resulting in an infection dose of ~10^5 cells per mouse. Additional cholestyramine was inoculated twice within 4 h by oral gavage. And they were anesthetized by inhalation of isoflurane gas every time before inoculation. To determine the exact inputs, appropriate dilutions of the inocula were plated on LB-Sm/X-Gal plates. After a 6-h period of colonization, the mice were euthanized by inhalation of carbon dioxide, and the small intestines from each mouse were collected by dissection. The small intestines were mechanically homogenized in LB broth with 15% glycerol, and appropriate dilutions were plated on LB-Sm/X-Gal. The dead animal bodies were processed by the staff of the animal facility according to the animal protocols that were approved by the Institutional Animal Care and Use Committee of Zhejiang A&F University.

Results

TcpP L152A Activating *toxT* Transcription in the Absence of Bile Salts

By performing the site-directed alanine mutagenesis of TcpP and detecting *toxT-lux* transcription activity, we

Table 1 Bacterial strains and primers used in this study

Strain	Relevant characteristics	Reference/source
V.cholerae strains		
C6706 Str^R	El Tor, streptomycin^R	[19]
MHV20	Δ*tcpP, tcpP* deletion	[11]
NLV2	Δ*tcpP*, (pBAD24, P_{BAD}-$tcpP_{WT}$)	This study
NLV3	Δ*tcpP*, (pBAD24, P_{BAD}-$tcpP_{I141A}$)	This study
NLV4	Δ*tcpP*, (pBAD24, P_{BAD}-$tcpP_{N142A}$)	This study
NLV5	Δ*tcpP*, (pBAD24, P_{BAD}-$tcpP_{V143A/V144A}$)	This study
NLV6	Δ*tcpP*, (pBAD24, P_{BAD}-$tcpP_{P145A/Y146A}$)	This study
NLV7	Δ*tcpP*, (pBAD24, P_{BAD}-$tcpP_{L147A/V148A}$)	This study
NLV8	Δ*tcpP*, (pBAD24, P_{BAD}-$tcpP_{F149A/S150A}$)	This study
NLV9	Δ*tcpP*, (pBAD24, P_{BAD}-$tcpP_{L152A}$)	This study
NLV10	Δ*tcpP*, (pBAD24, P_{BAD}-$tcpP_{Y153A}$)	This study
NLV11	Δ*tcpP*, (pBAD24, P_{BAD}-$tcpP_{V154A}$)	This study
NLV12	Δ*tcpP*, (pBAD24, P_{BAD}-$tcpP_{L156A/L157A}$)	This study
NLV13	Δ*tcpP*, (pBAD24, P_{BAD}-$tcpP_{P158A/V159A}$)	This study
NLV14	Δ*tcpP*, (pBAD24, P_{BAD}-$tcpP_{I160A/W161A}$)	This study
NLV15	Δ*tcpP*, (pBAD24, P_{BAD}-$tcpP_{W1162A/S163A}$)	This study
MHV41	Δ*dsbA* /Δ*tcpP, VcdsbA* and *tcpP* double deletion	[17]
YZV1	Δ*dsbA* /Δ*tcpP*, (pBAD24, P_{BAD}-$tcpP_{WT}$)	This study
YZV2	Δ*dsbA* /Δ*tcpP*, (pBAD24, P_{BAD}-$tcpP_{L152A}$)	This study
YZV3	Δ*tcpP*, (pBAD24, P_{BAD}-$tcpP_{L152A/C207S}$)	This study
YZV4	Δ*tcpP*, (pBAD24, P_{BAD}-$tcpP_{C207S}$)	This study
YZV5	Δ*tcpP*, P_{tcp}-$tcpP_{L152A}$	This study
Escherichia coli strains		
DH5α	*E. coli* for Cloning strain	[20]
BTH101	*E. coli* two-hybrid system detected strain	[21]
Cloning	Primer sequence (5′ → 3′)	
P_{BAD}-*tcpP*	F: GCGGAATTCATGGGGTATGTCCGCGTGAT	
	R: GCGCTGCAGCTAAAAATCGCTTTGACAGG	
TcpP-T25	F: GCGTCTAGAGATGGGGTATGTCCGCGTGAT	
	R: GCGGGTACCCTAAAAATCGCTTTGACAGGA	
TcpP-T18	F: GCGTCTAGAGATGGGGTATGTCCGCGTGAT	
	R: GCGGAATTCCTAAAAATCGCTTTGACAGGA	
P_{tcpP}-*tcpP*	F: GCGGAATTCGAGAATTACTTTCTGATATCG	
	R: GCGAGGCCTCAACGAATAGACCTGTAGCG	

found that TcpP alanine substitutions at amino acids 141 to 148 and 156 to 159 caused decreased an overall TcpP activity (Fig. 1a). The activity of TcpP WT was increased over sixfold in the presence of taurocholate, while the L152A mutant which activated *toxT* transcription to the same level as TcpP WT had significantly reduced fold increases in these experiments (Fig. 1b). We previously reported another TcpP mutant, TcpP C218S, also constitutively activates virulence gene expression in *V. cholerae* because an intermolecular disulfide bond between C207 in the periplasmic domain of TcpP could be constitutively formed in this mutant which results in the activation of virulence gene expression in the absence of stimuli [11].

It has been suggested that TcpP binds to the *toxT* promoter as a dimer [25]. And we previously reported that bile salts taurocholate enhances TcpP activity by stimulating TcpP homodimerization [11]. So, the L152A mutant activating *toxT* transcription in the absence of taurocholate might be the reason that the mutagenesis of L152A alters the dimerization of TcpP. We tested this hypothesis by examining TcpP L152A homodimerization using a bacterial adenylate cyclase two-hybrid system [21] in *E. coli*. We found

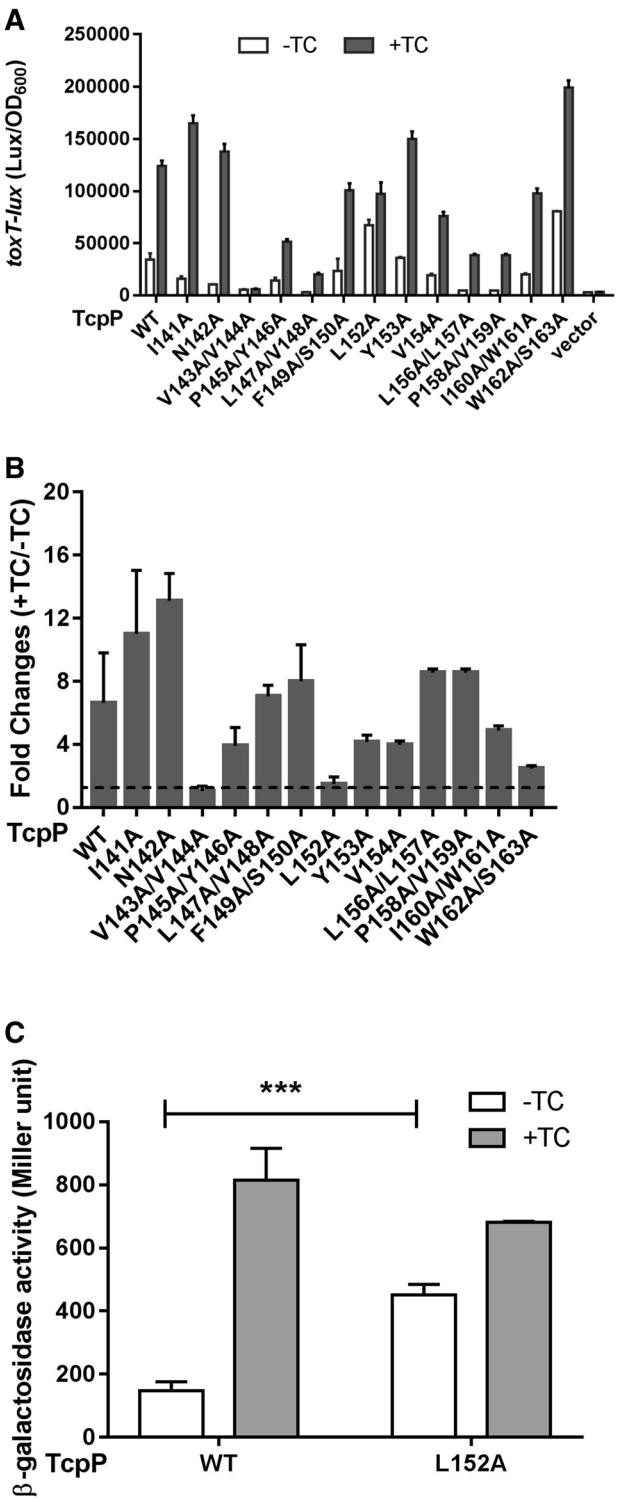

Fig. 1 TcpP L152A constitutively activates toxT transcription and forms homodimer in the absence of taurocholate (TC). **a** TcpP alanine mutants activating *toxT* transcription on response to TC. Luminescence of *V. cholerae ΔtcpP* containing P*toxT–luxCDABE* transcriptional fusion plasmids and pBAD vector control (vector) or pBAD-TcpP WT or alanine mutants was measured and reported as light units/OD_{600}. Data are mean and SD of three independent experiments. **b** The induction folds of TcpP WT and different mutants activating *toxT* transcription by TC according to the luminescence activity obtained in panel **a**. The dashed line represents onefold changes of TcpP WT or mutants with the addition of TC. TcpP mutant with results below the line represent decreased sensitivity to TC. **c** TcpP homodimerization assay by a bacterial two-hybrid system. Data are mean and SD of three independent experiments. ***$P < 0.001$ (Student *t* test)

that TcpP dimer formation was found to be constitutive in TcpP L152A (Fig. 1c). These results indicate that the residues within the transmembrane helix play an important role for TcpP transcription activity in response to bile salts.

TcpP L152A Needs C207 and DsbA to be Active

Previously published evidence has also shown that the intermolecular disulfide bond between C207 oxidized by DsbA is critical for TcpP transcription activity [11]. TcpP C207S mutant is completely inactive and TcpP WT is unable to activate *toxT* transcription in *V. cholerae dsbA* gene deletion mutant [11, 17]. To test if the transcription activity of TcpP L152A mutant also depends on the C207 and DsbA, we constructed TcpP L152A/C207S mutant and analyzed the transcription activity of this mutant by the same experiments as described above. We found that like TcpP C207S, TcpP L152A/C207S is unable to activate *toxT* transcription in *V. cholerae* (Fig. 2a). We then tested TcpP L152A activity in *V. cholerae dsbA* gene deletion mutant (*ΔdsbA*), and found that like TcpP WT, TcpP L152A cannot activate *toxT* transcription or TcpA production in *ΔdsbA*, either (Fig. 2b, c). These results suggest that even though TcpP L152A has the bile salt-independent activity, this activity still depends on the residue of C207 in TcpP periplasmic domain and DsbA has to be involved. The mutagenesis of L152A might change the conformation of TcpP which enable it to form homodimer and activate downstream gene expression in the absence of taurocholate; however, the homodimerization and the transcription activity of this mutant still need forming disulfide bond between C207 catalyzed by DsbA.

TcpP L152A Constitutively Activates Virulence Gene Expression

To further characterize the effect of TcpP L152A on *V. cholerae* infection, we generated *V. cholerae* TcpP L152A mutant by replacing *tcpP* WT with *tcpP* L152A in the chromosome expressed under the control of native *tcpP* promoter. This strain demonstrated increased *toxT* transcription activity and TcpA production in the absence of taurocholate (Fig. 3a, b). We then performed a competitive colonization assay by using infant mice, where the L152A mutant colonized with the same efficiency as WT strain when no bile salt-sequestering resin cholestyramine was used. However, the

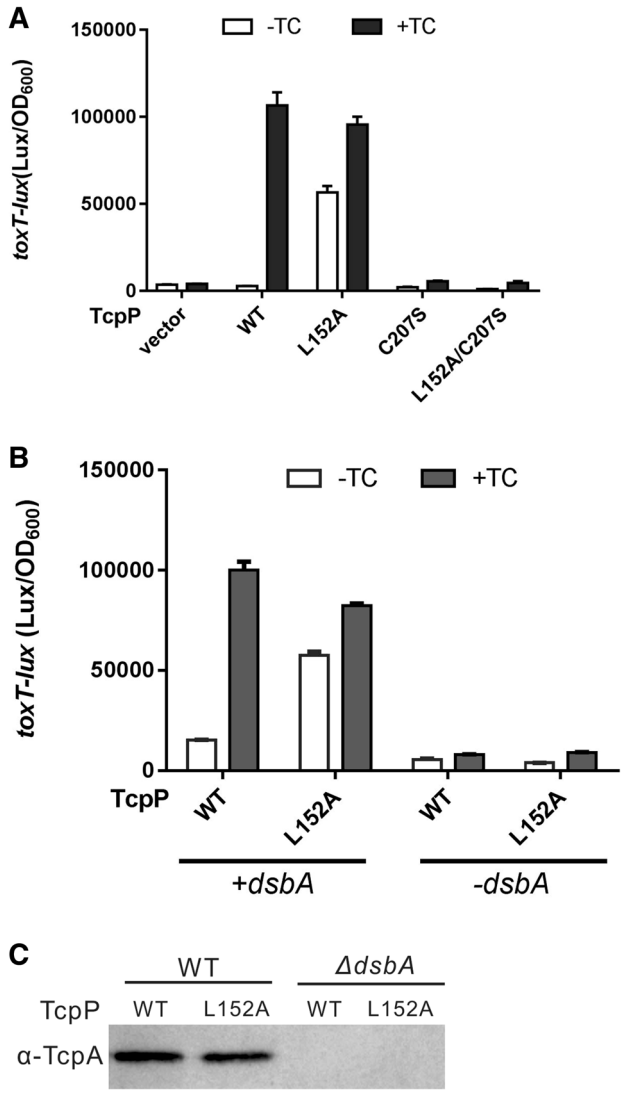

Fig. 2 TcpP L152A constitutively activating virulence gene expression depends on C207 in the periplasmic domain of TcpP and DsbA in *V. cholerae*. **a** Luminescence of *V. cholerae ΔtcpP* containing P*toxT–luxCDABE* transcriptional fusion plasmid and pBAD-TcpP WT or mutants was measured and reported as light units/OD_{600}. Data are mean and SD of three independent experiments. **b** Luminescence of *V. cholerae ΔtcpP* (+*dsbA*) or*ΔtcpP*/*ΔdsbA* (−*dsbA*) containing P*toxT–luxCDABE* transcriptional fusion plasmids and pBAD-TcpP WT or L152A were tested by the same methods as described above. Data are mean and SD of three independent experiments. **c** *V. cholerae ΔtcpP* (+*dsbA*) or *ΔtcpP*/*ΔdsbA* (-*dsbA*) containing pBAD-TcpP WT or L152A were grown in LB in the presence of 1 mM TC until $OD_{600} \approx 0.8$. 1 mg cell lysates were separated by SDS-PAGE and TcpA was detected by the Western blot using anti-TcpA antibody. Blot shown is representative of at least three separate experiments

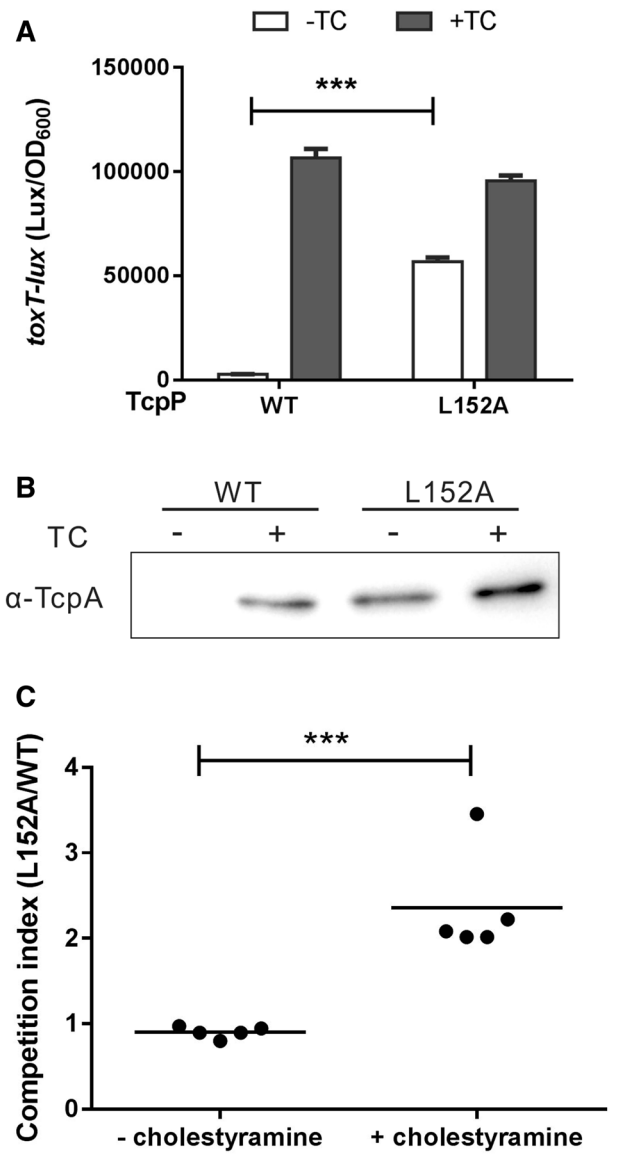

Fig. 3 TcpP L152A constitutively activating virulence gene expression confers *V. cholerae* colonization advantage in infant mouse. **a** *V. cholerae* WT or *tcpP* L152A mutant containing P*toxT–luxCDABE* transcriptional fusion plasmid were grown in LB in the presence (+) and absence (−) of 1 mM TC until $OD_{600} \approx 0.2$. Luminescence was measured and reported as light units/OD_{600}. Data are mean and SD of three independent experiments. **b** *V. cholerae* WT or *tcpP* L152A mutant were grown in LB in the presence (+) and absence (−) of 1 mM TC. 1 mg cell lysates was separated by SDS-PAGE and TcpA was detected by Western blotting using anti-TcpA antibody. Blot shown is representative of at least three separate experiments. **c** In vivo competition assay using an infant mouse model. Infant mice were inoculated with the mixture of TcpP L152A and WT with (+) or without (−) cholestyramine. After a 6-h period of colonization, intestinal homogenates were collected, and the ratio of L152A-to-WT bacteria was determined and normalized against input ratios. $^{***}P < 0.001$ (Student *t* test)

L152A mutant displayed a slight advantage in colonization with coadministration of the cholestyramine. These results suggest that inducible virulence expression is optimal, but that in the absence of a signal, constitutive expression of virulence confers an advantage.

Discussion

The ability of *V. cholerae* to colonize and cause disease in hosts requires production of a number of virulence factors during infection. Extensive studies show that coordinated expression of virulence genes results from the activity of a cascading system of regulatory factors by sensing to different environmental signals [4]. In previous studies, we found that bile salts and calcium ion present in the small intestine enhance virulence activation of *V. cholerae* by increasing Tcp–TcpP interaction. We found that bile salts enhance TcpP homodimerization by repressing periplasmic DsbA reoxidation which results in forming more intermolecular disulfide bonds (TcpP–TcpP) between C207 in the periplasmic domain of TcpP [11, 17], and calcium affects TcpP activity by altering protein movement in the inner membrane [18]. In this study, we identified a key amino acid in the transmembrane helix of TcpP that can alter the sensitivity of TcpP in response to its positive effectors. The mutagenesis of L152A enables TcpP to form homodimers and activate *toxT* transcription without bile salts induction. We previously reported another TcpP mutant, TcpP C218S, also constitutively activates virulence gene expression in *V. cholerae* because an intermolecular disulfide bond between C207 in the periplasmic domain of TcpP could be constitutively formed in this mutant which results in the activation of virulence gene expression in the absence of stimuli [11]. For TcpP L152A, we found that C207 has to be present in this mutant to be active. And the disulfide bond catalase DsbA is also indispensible for TcpP L152A activity. These might indicate that except for the role that the transmembrane helix helps TcpP to localize into the membrane, the residues in this helix could affect the protein conformation of TcpP, especially the conformation of the periplasmic domain, so as to change the way in which the disulfide bonds form between the cysteine residues in the periplasmic domain and subsequently change TcpP transcription activity in response to host stimuli, like bile salts.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

Ethics Approval All procedures performed in studies involving animals were in accordance with the ethical standards of the Institutional Animal Care and Use Committee of Zhejiang A&F University at which the studies were conducted. (Permit No. ZJAFU/IACUC_2011-10-25-02.)

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注：以上检索结果均得到被检索人的确认。

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检索人：桑宇芳

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