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Redox pathway sensing bile salts activates virulence gene expression in *Vibrio cholerae*

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Summary

***Vibrio cholerae*, the causative agent of the severe diarrheal disease cholera, has evolved signal transduction systems to control co-ordinately the expression of virulence determinants. It was previously shown that the presence of the bile salts glycocholate and taurocholate in the small intestine causes dimerization of the transmembrane transcription factor TcpP by inducing intermolecular disulphide bonds in the TcpP periplasmic domain. In this study, they further investigated the mechanism of how taurocholate affects *V. cholerae* virulence determinants. *In vitro* assay of TcpP oxidation by VcDsbA showed that VcDsbA induced TcpP dimerization in the presence of taurocholate. Taurocholate bound to VcDsbA with a K_D of 40 ± 2.5 μM, and also bound other Dsb proteins, including EcDsbA, EcDsbC and VcDsbC. Taurocholate inhibited VcDsbA reductase activity without affecting VcDsbA secondary structure or thermostability. VcDsbA and its substrates were more extensively reduced in the presence of taurocholate, as compared with their redox state in the absence of taurocholate. The data presented here not only provide new insights into the mechanism by which bile salts induce *V. cholerae* virulence but also suggest a means by which to develop inhibitors against DsbA.**

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Introduction

Vibrio cholerae is a Gram-negative, facultative pathogen and is the causative agent of cholera. The *Vibrio* life cycle begins with a free swimming phase in aquatic environments. Human infection normally starts with the ingestion of food or water contaminated with *V. cholerae*. *Vibrio cholerae* produces an array of virulence factors when it colonizes the host (Miller *et al.*, 1987; Herrington *et al.*, 1988). An *ex vivo* intestinal model revealed that *in vivo* virulence gene expression is dependent upon both an anaerobic environment and on chemicals present in the small intestine, including bile salt (Yang *et al.*, 2013).

The Lys-R type activator AphB activates *tcpP* expression under anaerobic conditions at neutral pH (Liu *et al.*, 2011; Kovacikova *et al.*, 2010). The AphB crystal structure suggests that residues in the ligand-binding pocket of AphB influence its ability to respond to both intracellular pH as well as to anaerobiosis (Taylor *et al.*, 2012). Activated by AphB under anaerobic conditions, TcpP then activates *toxT* expression together with ToxR in the presence of bile salts secreted into the small intestine from the gallbladder. TcpP is a transmembrane protein with two cysteine residues in its periplasmic domain and the redox status of these two cysteines are critical to TcpP's activation of *toxT*. Bile salts induce intermolecular TcpP disulphide bond formation to activate virulence gene expression (Yang *et al.*, 2013). However, the detailed mechanism of how bile salts induce TcpP intermolecular disulphide bond is not yet clear.

DsbA, one of the best characterized oxidoreductases, participates in protein folding by introducing disulphide bonds into proteins secreted to the periplasm (Depuydt

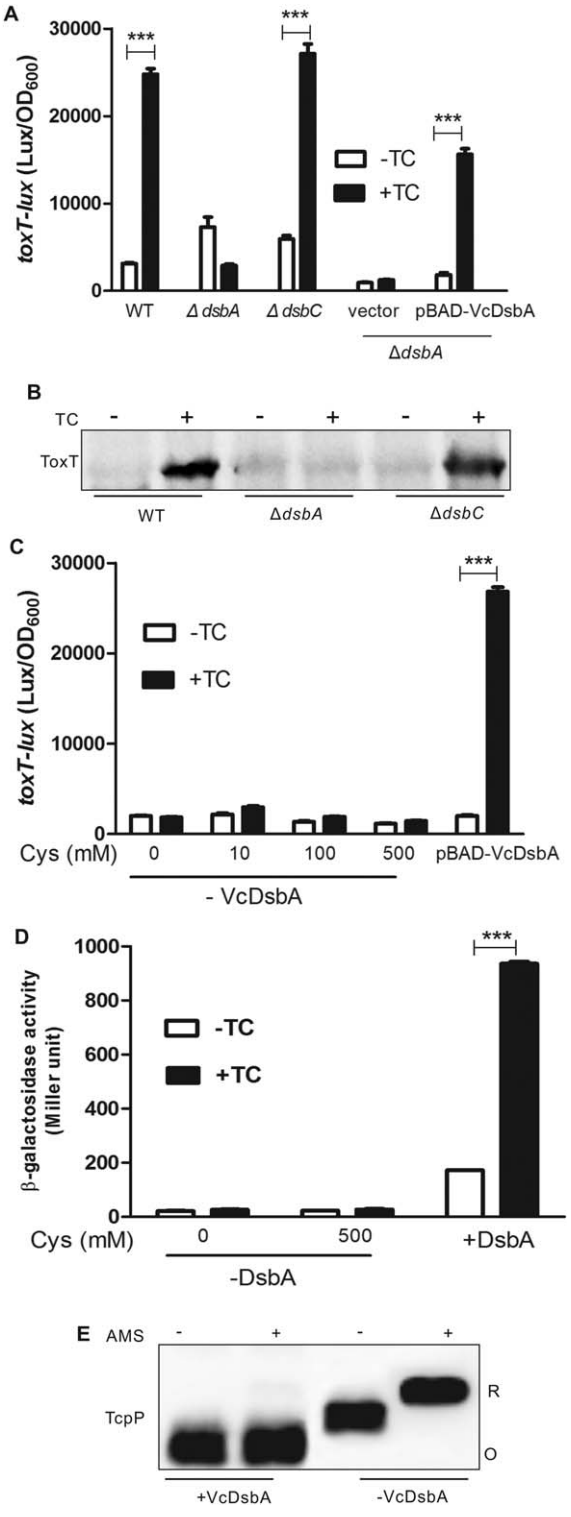

Fig. 1. VcDsbA is indispensible for virulence gene expression in the presence of TC.
A. *V. cholerae* strains WT, Δ*dsbA*, and Δ*dsbC* containing P_{toxT}-*lux* transcriptional fusion plasmids or Δ*dsbA* containing P_{toxT}-*lux* reporter and either P_{BAD} vector control or P_{BAD}-*VcdsbA* were grown in LB with or without 0.01% arabinose in the presence or absence of 1 mM TC at 37°C until $OD_{600} \approx 0.2$. Luminescence was measured and reported as light units/OD_{600}. Data are the means ± SD ($n = 3$). ***, $P < 0.0001$.
B. ToxT Western blot of *V. cholerae* strains WT, Δ*dsbA*, and Δ*dsbC* grown in LB in the presence or absence of 1 mM TC for 6 h.
C. Δ*dsbA* containing P_{toxT}-*lux* reporter and P_{BAD} vector control was grown in M9 medium with different concentrations of cysteine as indicated in the presence or absence of 1 mM TC at 37°C until $OD_{600} \approx 0.2$. Luminescence was measured and reported as light units/OD_{600}. Δ*dsbA* containing P_{toxT}-*lux* reporter and P_{BAD}-*VcdsbA* was grown and measured as described above. Data are means ± SD ($n = 3$). ***, $P < 0.0001$.
D. TcpP dimerization assay in bacterial two-hybrid system. Full-length TcpP was 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 (Karimova *et al.*, 1998) or *cyaA*/*dsbA* double mutants (Lippa and Goulian, 2012). Cultures were grown in M9 medium with different concentrations of cysteine as indicated in the presence or absence of 1 mM TC at 30°C for 8 h without shaking and β-galactosidase activity was measured and reported as Miller Units (Miller, 1972). Data are means ± SD ($n = 3$). ***, $P < 0.0001$. **E.** AMS trapping assay. *V. cholerae* Δ*tcpP and* Δ*tcpP*/*dsbA* containing a plasmid expressing $TcpP_{125\text{-}221}$ (cytoplasmic domain of TcpP was removed to avoid interference of cysteine residues in cytoplasmic domain) using the P_{BAD} promoter were induced 10 min before AMS acid trapping. Reduced TcpP shows a 1-kD upshift on SDS-PAGE. TcpP was detected by Western blotting.

et al., 2011). DsbA was reported to be essential for the correct folding of secreted disulphide-containing bacterial proteins, including many virulence factors (Heras *et al.*, 2009). VcDsbA was first characterized for its role in the functional maturation of secreted virulence factors in *V. cholerae*, including cholera toxin and toxin-coregulated pili (TCP), and has been identified as a potential target for the development of antibacterial compounds (Peek and Taylor, 1992; Hu *et al.*, 1997; Heras *et al.*, 2009). VcDsbA is as oxidizing as EcDsbA, and the binding mode identified between *Escherichia coli* DsbB and DsbA is likely to be conserved in VcDsbA (Walden *et al.*, 2012). The VcDsbA crystal structure revealed two small-molecule binding regions (Walden *et al.*, 2012).

In this study, we investigated the effect of the bile salt taurocholate on VcDsbA activity *in vivo* and *in vitro*. We found that that taurocholate not only can bind VcDsbA directly *in vitro*, but also can inhibit VcDsbA reductase activity without affecting VcDsbA structure. *In vivo* assays showed that taurocholate changes the redox status of VcDsbA and its substrates. We speculate that bile salts induce *V. cholerae* virulence gene expression by interfering with the redox reaction of VcDsbA in the periplasm of bacteria.

Results

VcDsbA oxidizes TcpP in vivo

VcDsbA, a disulphide bond protein in the *V. cholerae* periplasm is essential to *V. cholera* pathogenesis

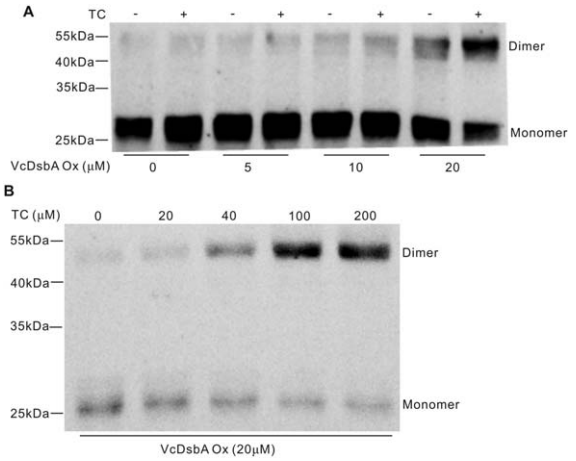

Fig. 2. VcDsbA oxidizes TcpP *in vitro*.
A. The membrane purified from *E. coli* Δ*dsbA* (Lippa and Goulian, 2012) containing a plasmid expressing $TcpP_{C19S/C124S}$ was mixed with VcDsbA Ox in the presence or absence of TC.
B. The$TcpP_{C19S/C124S}$ membrane containing fixed concentrations of VcDsbA Ox was mixed with different concentrations of TC.
Samples in (A) and (B) were incubated at 30°C for 30 min and then different forms of TcpP were separated by SDS-PAGE and TcpP was detected by Western blot using anti-FLAG antibody.

because of its role in the functional maturation of secreted virulence factors (Peek and Taylor, 1992). We found that VcDsbA not only helps the folding of virulence proteins, it is also indispensable for the expression of the virulence master regulator, ToxT, in the presence of taurocholate (TC) (Fig. 1A and B). TC activates *V. cholerae* virulence by inducing TcpP intermolecular disulphide bonds between the two cysteine residues in the periplasmic domain, in a process likely mediated by VcDsbA (Yang *et al.*, 2013). We found that TC was not able to induce *toxT* expression or TcpP dimerization in the absence of DsbA, even with additional cysteine in the growth medium (Fig. 1C and D). To confirm whether the two cysteines in the periplasmic domain of TcpP were oxidized by VcDsbA *in vivo* and to avoid interference of cysteine residues in cytoplasmic domain, a truncated TcpP mutant containing the 125th to 221st residues of the C-terminal was used for AMS trapping assay. Results from the assays confirmed that TcpP is the substrate of VcDsbA (Fig. 1E). TC had no impact on VcDsbA expression levels (Supporting Information Fig. S1).

VcDsbA induces TcpP homodimerization in vitro

To determine whether TC induces TcpP homodimerization by directly involving in TcpP oxidization by VcDsbA, we purified the membrane contained $TcpP_{C19S/C124S}$-cFLAG overexpressed under an arabinose-inducible promoter. This TcpP mutant localizes to the membrane (Supporting Information Fig. S2A) and has the same phenotype as $TcpP_{wt}$ (Supporting Information Fig. S2B), but is less sensitive to oxygen and is more easily studied in redox reactions. $TcpP_{C19S/C124S}$ was mixed with $VcDsbA_{Ox}$ in the presence or absence of TC, separated by SDS-PAGE, and TcpP was detected by Western blotting. Oxidized VcDsbA in the presence of TC induced $TcpP_{C19S/C124S}$ homodimerization (Fig. 2A). TcpP homodimerization increased as a function of TC concentration in the presence of a fixed amount of $VcDsbA_{Ox}$ (Fig. 2B). No dimerization was observed with the $TcpP_{C19S/C124S/C207S/C218S}$-cFLAG mutant, suggesting that VcDsbA can catalyze intermolecular disulphide bond formation in the TcpP periplasmic domain (Supporting Information Fig. S2C). These results suggest that TC enhances TcpP homodimerization probably by affecting VcDsbA oxidation of TcpP.

Bile salts bind VcDsbA in vitro

To differentiate whether TC directly affects VcDsbA or TcpP, we investigated the interaction between TC and the proteins by using isothermal titration calorimetry (ITC). We first tested the binding of VcDsbA to TC. An exothermic reaction was observed when titrating TC into VcDsbA (Fig. 3A). Analysis of the raw data using a 1:1 binding model revealed a binding affinity K_D of 40 ± 2.5 μM with an enthalpic contribution ($\Delta H = -3.0 \pm 0.1$ kcal/mol) and an entropy of binding ($\Delta S = 9.9$ cal/mol/deg). When $VcDsbA_{C30A}$, $VcDsbA_{C32A}$ or $VcDsbA_{C30A/C32A}$ were studied, similar binding curves of TC were observed under the same conditions (Fig. 3B–D). Thus, the cysteine residues in the CXXC motif are not required for bile salt binding and TC does not bind to the VcDsbA substrate binding site. To identify if binding was specific or non-specific, we also tested TC binding to VcDsbC, EcDsbA, EcDsbC and EcTrxA (Holmgren, 1979) from *Escherichia coli*, and MtDsbA from *Mycobacterium tuberculosis* (Chim *et al.*, 2013). TC bound VcDsbC, EcDsbA and EcDsbC with a K_D of 176 ± 2, 146 ± 7 and 143 ± 2 μM, respectively (Fig. 3E–G). There was no evidence of TC binding to EcTrxA or MtDsbA (Supporting Information Fig. S3A and B). The molar ratio of each protein-TC binding is close to 1, except for VcDsbC and EcDsbC, which is 2, probably because VcDsbC and EcDsbC remain as dimers (Banaszak *et al.*, 2004, Jiao *et al.*, 2013). We also tried to test the interaction of TcpP with TC by ITC. TcpP was purified as a membrane protein in the presence of 0.1% Triton X-100. However, there was no evidence of TC binding to TcpP (Supporting Information Fig. S3C).

Bile salts inhibit VcDsbA reductase activity in vitro

To investigate if the direct interaction of TC and VcDsbA alters VcDsbA activity, we tested VcDsbA for its ability

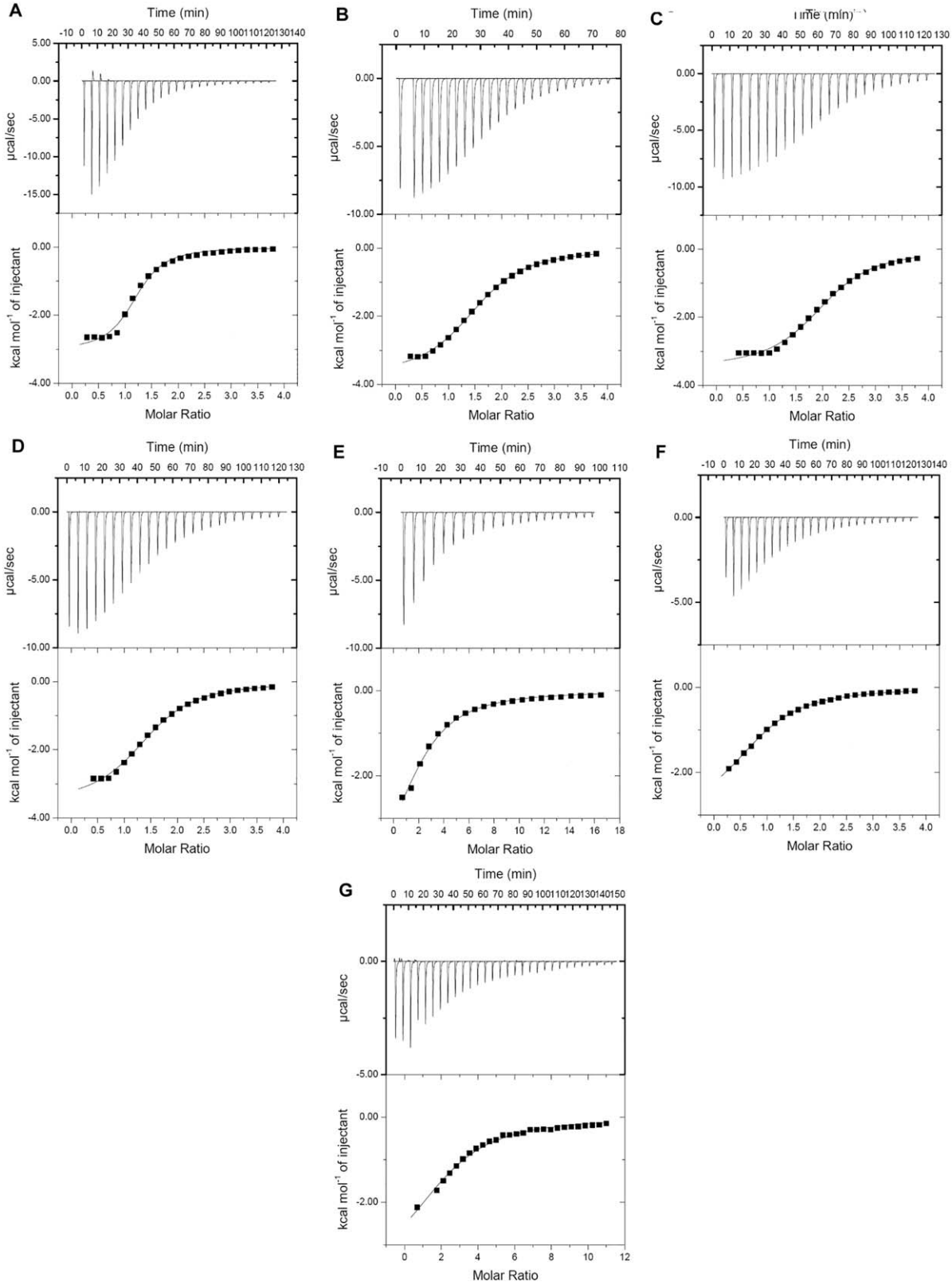

Fig. 3. TC interacts with Dsb proteins.
A–G. ITC data titrating 4 mM of TC into 0.2 mM of each protein. (A) VcDsbA. (B) VcDsbA$_{C30A}$. (C) VcDsbA$_{C32A}$. (D) VcDsbA$_{C30A/C32A}$. (E) VcDsbC. (F) EcDsbA. (G) EcDsbC. All data show a representative example from three replicates.

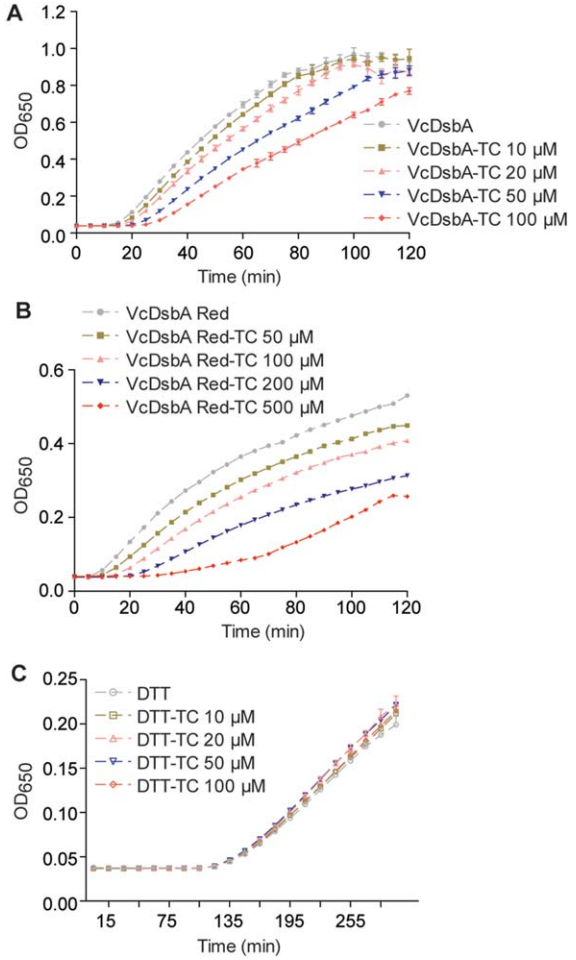

Fig. 4. TC inhibits VcDsbA reductase activity.
A. Insulin (170 μM) and 0.33 mM DTT were incubated with 5 μM VcDsbA, with differing concentrations of TC. The reduction of insulin was measured by monitoring the increase in absorbance at OD_{650}.
B. Insulin (170 μM) was incubated with 50 μM $VcDsbA_{Red}$, with differing concentrations of TC.
C. Insulin (170 μM) was incubated with differing concentrations DTT. The reduction of insulin was measured as described above. All the data shown are averages of three independent experiments.

to catalyze the reduction of insulin, in a standard reductase activity assay (Holmgren, 1979). Under the standard assay conditions, VcDsbA catalyzes insulin reduction by dithiothreitol (DTT). This results in precipitation of the B chain of insulin and a consequent increase in the OD_{650}. We found that increasing TC concentrations caused a reduction in VcDsbA catalysis rates (Fig. 4A). The mechanism of the thioredoxin-catalyzed reduction of insulin with DTT (Reaction 1) is suggested to be analogous with the mechanism for thioredoxin reductase-catalyzed disulphide reduction (Reactions 2 and 3) (Holmgren, 1979):

$$\text{Dithiothreitol}-(SH)_2 + \text{insulin}-S_2 \xrightarrow{\text{DsbA}} \text{Dithiothreitol}-S_2 + \text{insulin}-(SH)_2 \quad (1)$$

$$\text{DsbA}-S_2 + \text{Dithiothreitol}-(SH)_2 \rightleftarrows \text{Dithiothreitol}-S_2 + \text{DsbA}-(SH)_2 \quad (2)$$

$$\text{Insulin}-S_2 + \text{DsbA}-(SH)_2 \rightleftarrows \text{Insulin}-(SH)_2 + \text{DsbA}-S_2 \quad (3)$$

To determine which reaction, (2) or (3), was inhibited by TC, insulin was reduced with either DTT or $VcDsbA_{Red}$ in the presence or absence of TC. We found that TC inhibited only the reduction of insulin by $VcDsbA_{Red}$ and did not affect insulin reduction by DTT (Fig. 4B and C), indicating that TC selectively inhibits VcDsbA reductase activity. We also tested EcDsbA and EcTrxA reductase activity in the presence or absence of TC, and found that TC also inhibited EcDsbA reductase activity (Supporting Information Fig. S4A), but had no effect on EcTrxA (Supporting Information Fig. S4B).

Bile salts do not alter VcDsbA structure or redox potential

High bile salt concentrations can induce protein unfolding and aggregation (Cremers *et al.*, 2014). To eliminate the possibility that the impact of TC on VcDsbA activity might be due to changes in protein conformation, we incubated 10 μM of either $VcDsbA_{Red}$ or $VcDsbA_{Ox}$ with 100 μM of TC, and determined VcDsbA secondary structures by using circular dichroism (CD) spectroscopy. Secondary structures were unaffected by TC concentrations (Fig. 5A and B). We also investigated the relative stability of the two redox forms of VcDsbA in the presence or absence of TC by observing changes in CD during thermal unfolding of reduced and oxidized forms of the enzyme over the temperature range 25°C–105°C at pH 7.0 (Fig. 5C). The thermal transition values calculated from these data showed that the melting temperatures of the two redox forms of VcDsbA are in agreement with previous data for VcDsbA (Walden *et al.*, 2012), and no significant changes were observed as a function of TC.

We then determined if TC would affect the redox potential of VcDsbA *in vitro*. Wild-type DsbA was incubated with different glutathione redox buffers, and the redox state of the proteins at equilibrium was measured from the calculated fraction of reduced VcDsbA at different concentrations of $[GSH]^2/[GSSG]$ with or without TC (Fig. 5D). The curves from the nonlinear

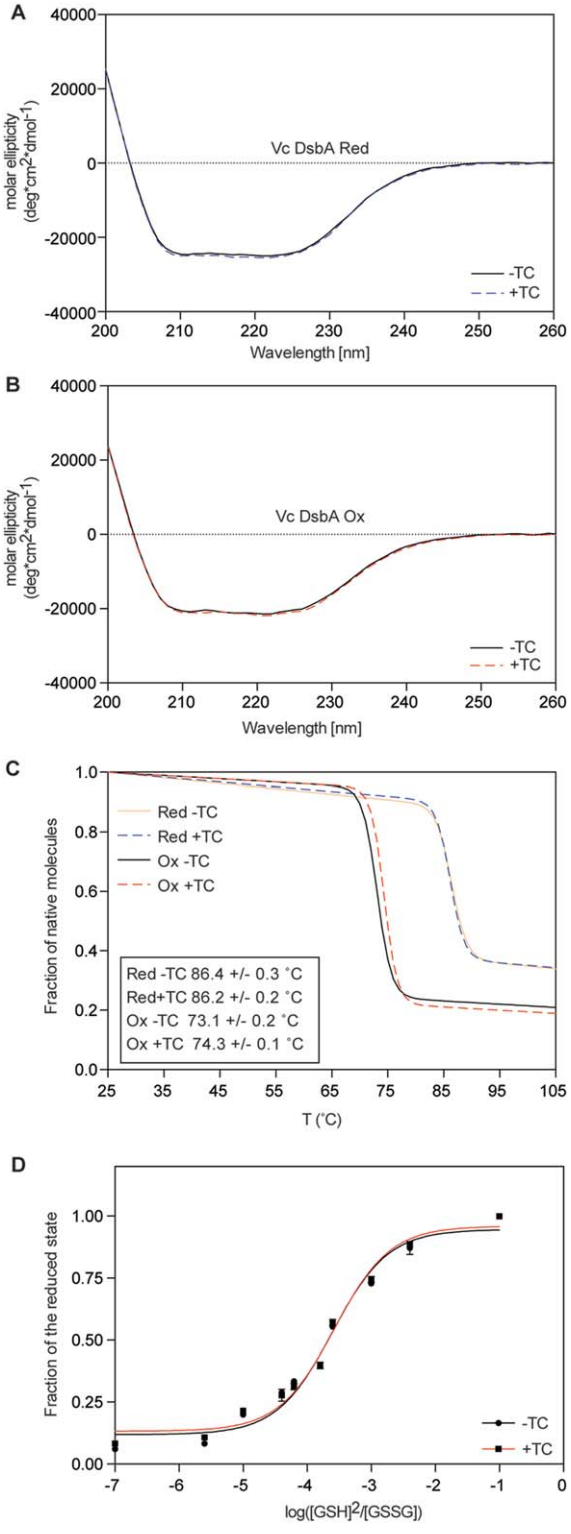

Fig. 5. TC does not alter VcDsbA structure *in vitro*.
A and B. Influence of TC on the secondary structure of $VcDsbA_{Red}$ (A) and $VcDsbA_{Ox}$ (B) at 30°C. Proteins (10 μM) were incubated in the absence (solid line) or in the presence of 100 μM TC (dashed line) for 1 h. Then, far-UV–CD spectra were recorded at the indicated temperature. All spectra are buffer-corrected.
C. Relative stability of reduced and oxidized VcDsbA. The plot shows the thermal unfolding of VcDsbA measured in 100 mM sodium phosphate and 1 mM EDTA at pH 7.0 with 10 μM oxidized or reduced VcDsbA in the presence of absence of 100 μM TC.
D. Characterization of the redox potential of VcDsbA in the presence of TC. Non-linear fit to the fraction of reduced VcDsbA at different ratios of reduced:oxidized glutathione. The curve was fit to the averaged data from three replicate experiments (the mean and standard deviation of each point are shown).

fitting to the fraction of reduced VcDsbA at different ratios of reduced:oxidized glutathione were not different as a function of TC, indicating that TC does not alter VcDsbA redox potential *in vitro*.

Bile salts change VcDsbA redox status in vivo

To determine whether TC affects VcDsbA activity *in vivo*, we first investigated the redox status of VcDsbA as a function of TC. We observed an increased percentage of reduced VcDsbA in the presence of TC, while VcDsbA was almost completely oxidized in the absence of TC (Fig. 6A and B). DsbA reductase activity correlates with DsbA reoxidation by DsbB (Ren *et al.*, 2009). We found that VcDsbA oxidization by the membrane protein VcDsbB was also inhibited by TC *in vitro* (Fig. 6C). Reduced VcDsbA was totally oxidized by $VcDsbB^{+}$ membrane in less than 15 min while the oxidization is much slower in the presence of TC under the same conditions (Fig. 6C).The efficiency of VcDsbA oxidization by VcDsbB membrane *in vitro* decreased as a function of TC concentration in the presence of a fixed amount of VcDsbB while the amount of oxidized VcDsbA remained the same without VcDsbB, regardless of TC (Fig. 6C), indicating that TC inhibits the reoxidization of VcDsbA by VcDsbB *in vivo*.

Cadmium sensitivity is an indicator of oxidase capacity due to the high affinity of Cd^{2+} for protein thiol groups (Vallee and Ulmer, 1972). We then tested the cadmium-sensitivity of *V. cholerae*, as a function of VcDsbA expression. Consistent with the inhibition of VcDsbA reoxidization efficiency by TC, we observed that *V. cholerae* was more sensitive to cadmium in the presence, than in the absence of TC (Fig. 6D). TC may generally inhibit disulphide bond formation in the *V. cholerae* periplasm, including Dsb proteins such as DsbC, DsbB or their substrates, since the *dsbA* knock-out strain or the strain containing a VcDsbA CXXA mutant were also more sensitive to cadmium in the presence of TC (Fig. 6D).

Increased amounts of reduced EcDsbA *in vivo* facilitated EcDsbA to catalyze the folding of proteins with complex disulphide bond connectivities (Ren and Bardwell, 2011). To test if TC altered VcDsbA isomerase activity *in vivo*, we generated a selection system in *V. cholerae* that allowed us to link correct disulphide bond formation to bacterial antibiotic resistance. To do this, we engineered an additional nonconsecutive

disulphide into the ampicillin-resistance protein β-lactamase (a "PDI detector") (Ren and Bardwell, 2011). Both the ampicillin-sensitive phenotype of Δ*dsbC* strain and the ampicillin-resistant phenotype of VcDsbC complementation strain containing the PDI detector were observed (Fig. 6E), indicating that the PDI detector was functional in *V. cholerae*. We then tested the ampicillin resistance of the Δ*dsbC* strain as a function of VcDsbA complementation. No difference in resistance was observed as a function of incubation with TC, suggesting that TC did not alter VcDsbA isomerase activity (Fig. 6E). Ampicillin resistance was slightly enhanced in the wild type, Δ*dsbA*, and the VcDsbC complementation of Δ*dsbC* strains, suggesting that TC may inhibit disulphide bond formation in the periplasm of *V. cholerae*.

Bile salts affect the redox status of VcDsbA substrates in vivo

We next investigated the redox status of the VcDsbA substrate TcpP. TcpP can form intra- and inter-molecular disulphide bonds *in vivo* (Yang *et al*., 2013), affecting its dimerization state. There are four cysteine residues in the cytoplasmic domain of TcpP that probably remain in the reduced form. To differentiate the reduced and oxidized forms of TcpP, an *in vivo* differential thiol trapping approach with iodoacetamide and AMS was used (Leichert and Jakob, 2004). The method is based on the fact that both iodoacetamide and AMS react only with free thiol groups. First, *V. cholerae* Δ*tcpP* producing TcpP–cFLAG was labelled with iodoacetamide after arabinose induction in the presence or absence of TC. Subsequently, free iodoacetamide was removed, and all disulphide bonds were reduced by treatment with DTT. Free thiol groups were labelled with AMS in a second step. As a consequence, only cysteines that are present in an oxidized form and thus protected from iodoacetamide labelling in the first step are labelled with AMS, resulting in a detectable increase in molecular weight. We found that in the presence of TC, more reduced TcpP was observed at the beginning of arabinose induction (Fig. 7A and B). Increased TcpP expression did not alter the percentage of reduced TcpP, regardless of TC. Another VcDsbA substrate, the mutated β-lactamase containing four consecutive cysteine residues, remained primarily in the reduced form in the presence of TC (Fig. 7C and D). These data suggest that TC inhibits VcDsbA substrate oxidization *in vivo*, probably by decreasing the efficiency of VcDsbA reoxidization.

Discussion

Vibrio cholerae coordinates the transcription of virulence genes by sensing different environmental signals (Matson *et al*., 2007; Krukonis and DiRita, 2003). We previously reported that the bile salt TC activates *V. cholerae* virulence in the small intestine by inducing TcpP intermolecular disulphide bond formation, probably through VcDsbA (Yang *et al*., 2013).

Bile salts are synthesized in the liver from cholesterol in a multi-enzyme process (Begley *et al*., 2005). Preceding secretion into the small intestine, bile acids are conjugated as N-acyl amidates with either glycine or taurine (Hofmann, 1994). Bile salt concentrations are on the order of 10 mM in bile (Hofmann and Eckmann, 2006), but much of it is re-absorbed by the intestinal epithelium. Levels of deoxycholate of up approximately 0.8 mM have been measured in colonic water of human subjects consuming a high fat diet (Stadler *et al*., 1988), and thus the TC concentrations that are effective in inducing virulence gene expression (0.1–5 mM) are within a physiological range. In prokaryotes, bile salts in the concentration range of 0.1–10 mM have been implicated in regulating gene expression (Provenzano *et al*., 2000; Rosenberg *et al*., 2003; Hung and Mekalanos, 2005; Hung *et al*., 2006), membrane integrity (Merritt and Donaldson, 2009), toxin production (Hung and Mekalanos, 2005; Yang *et al*., 2013), protein conformational changes (Rosenberg *et al*., 2003; Cremers *et al*., 2014), and biofilm formation (Hay and Zhu, 2015; Hung *et al*., 2006). We found here that physiological concentrations of bile salts induce virulence in *V. cholerae* by interfering with the redox reaction of Dsb proteins in the periplasm.

We found that TC could bind several Dsb proteins from the Gram-negative bacteria *in vitro*, including VcDsbA, EcDsbA, VcDsbC and EcDsbC, but not EcTrxA or MtDsbA. CD spectroscopy assays showed that bile salts do not affect VcDsbA secondary structure or thermostability. TC binding also had no impact on VcDsbA redox potential. Two major factors affect TRX protein activities, redox properties, and substrate binding. XX residues from CXXC mainly affect the redox potential and pKa of the active site. The cis-Pro motif affects both substrate binding and redox properties. Given that TC binding has dramatic effects on VcDsbA activity without affecting redox potential, one might speculate that TC binding may affect the cis-Pro motif conformation (Ren *et al*., 2009; Paxman *et al*., 2009). NMR could be employed in the future to determine how bile salts interact with VcDsbA (Skinner and Laurence, 2008; Horvath *et al*., 2012).

In vitro VcDsbA functional assays show that in the presence of TC, VcDsbA enhances TcpP dimer formation while TC inhibits VcDsbA reduction of insulin. TC also inhibits VcDsbA reoxidization by VcDsbB *in vitro*. We speculate that TC affects VcDsbA activity by direct interaction with VcDsbA. TC interacts with VcDsbA and inhibits the reductase activity *in vitro* but does not denature the protein. TC did not alter VcDsbA redox status *in*

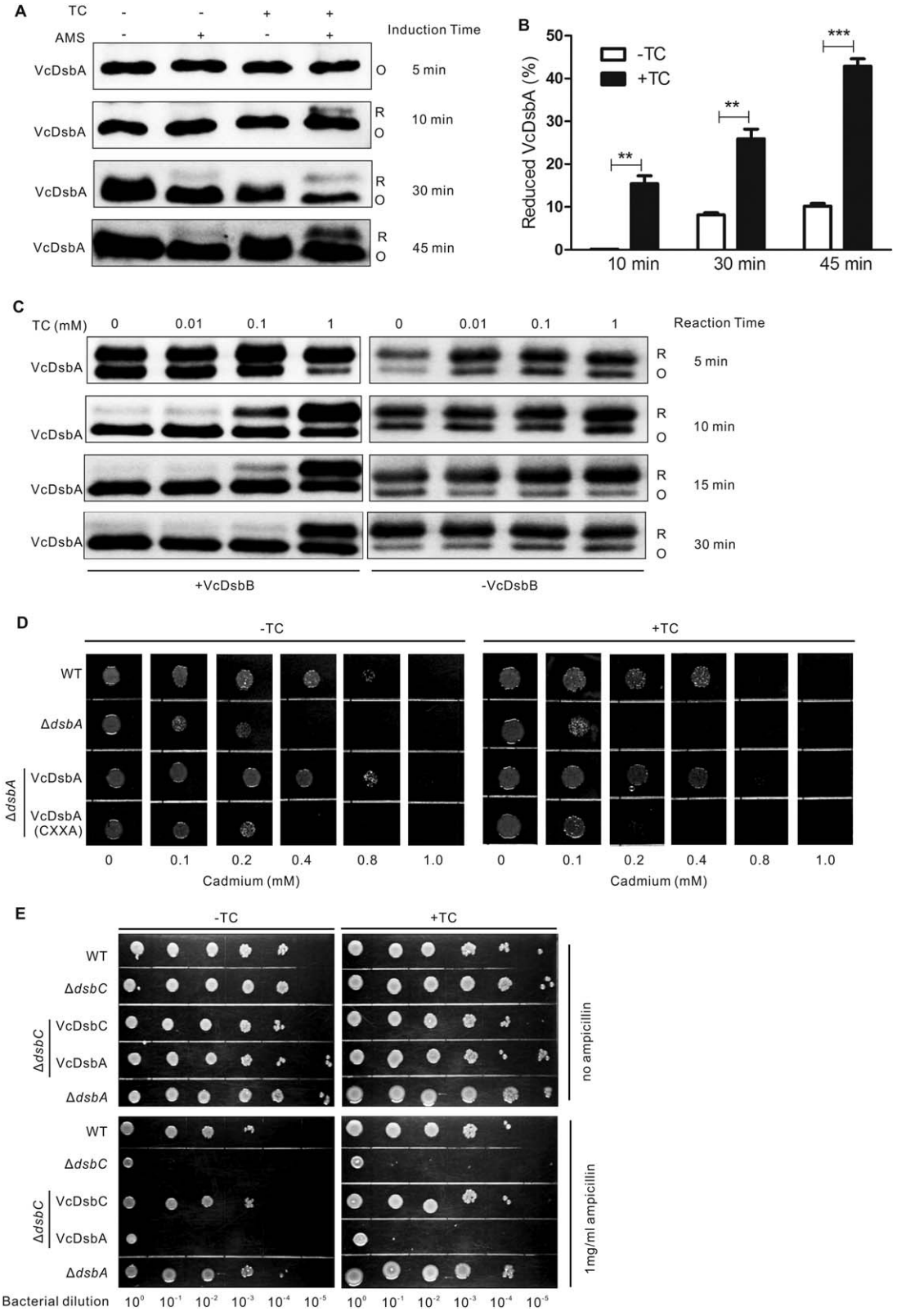
A
TC
AMS
Induction Time
VcDsbA
O
5 min
R
O
10 min
30 min
45 min
B
Reduced VcDsbA (%)
-TC
+TC
**
**
***
10 min
30 min
45 min
C
TC (mM)
0
0.01
0.1
1
Reaction Time
5 min
10 min
15 min
30 min
+VcDsbB
-VcDsbB
D
-TC
+TC
WT
ΔdsbA
ΔdsbA
VcDsbA
VcDsbA (CXXA)
0
0.1
0.2
0.4
0.8
1.0
Cadmium (mM)
E
-TC
+TC
WT
ΔdsbC
ΔdsbC
VcDsbC
VcDsbA
ΔdsbA
no ampicillin
1mg/ml ampicillin
Bacterial dilution
10^0
10^-1
10^-2
10^-3
10^-4
10^-5

Fig. 6. TC affects VcDsbA activity *in vivo*.
A. VcDsbA redox status assay by AMS trapping with the final concentrations of 1 mM of TC. Expression of VcDsbA was induced different time as indicated before AMS trapping. Reduced DsbA shows a 1 kDa upshift on SDS-PAGE. VcDsbA was detected by Western blot using anti-VcDsbA antibody. Blot shown is representative of at least three separate experiments.
B. Quantification of band intensities from blot shown in panel A was performed using ImageJ software. Graph represents percentages of reduced VcDsbA. Data shown are averages of three independent experiments. ***, $P < 0.0001$ and **, $P < 0.001$.
C. Reduced VcDsbA oxidized by VcDsbB present in the membranes *in vitro*. Reduced VcDsbA (2 μM) was incubated with or without DsbB-containing membranes and trapped with AMS after various incubation times. After incubation with $DsbB^+$ membrane, VcDsbA (O) shifts to a lower molecular weight band. VcDsbA was detected by Western blot using anti-VcDsbA antibody.
D. *V. cholerae* WT, Δ*dsbA* or Δ*dsbA* complemented with either VcdsbA or VcDsbA CXXA were tested in cadmium sensitivity assays by spotting 10^3 CFUs of each bacterial strain onto LB containing increasing concentrations of cadmium (0–1 mM) in the presence or absence of 1 mM TC.
E. *V. cholerae* WT, Δ*dsbC*, Δ*dsbA* or Δ*dsbC* complemented with either VcDsbC or VcdsbA were tested in ampicillin sensitivity assays using the PDI detector. Spot titrations were performed on LB plates containing 1 mg/mL ampicillin in the presence or absence of 1 mM TC for various strains transformed with the PDI detector construct.

vitro; however, VcDsbA and its substrates stay more in the reduced form *in vivo* with TC. These results indicate that TC interferes with the redox reaction between VcDsbA and substrates, such as insulin, VcDsbB, TcpP and β-lactamase, while TC does not alter redox reactions between VcDsbA and small molecules such as DTT, GSH or GSSG.

We suggest a working model of how VcDsbA induces TcpP intermolecular disulphide bond in the presence of TC (Fig. 8). In this model, the two cysteine residues in the periplasmic domain of TcpP are quickly oxidized by VcDsbA to form inhibitory intramolecular disulphide bonds in the absence of TC (Fig. 8A). Disulphide bonds oxidized by DsbA would tend to be formed between cysteines that appear sequentially in the polypeptide when it is transported to the bacterial inner membrane or to the periplasm (Kadokura and Beckwith, 2009). However, in the host intestine, bile salts interfere with redox reactions in the periplasm by interacting with VcDsbA. This interaction eventually leads to slowed VcDsbA reoxidation by VcDsbB, and VcDsbA is only partially in the oxidized form. A portion of the newly synthesized TcpP may escape from VcDsbA oxidation. The reduced form of TcpP then interacts with oxidized TcpP (intramolecular S—S) with or without catalysis from VcDsbA to form intermolecular disulphide bonds, which helps to maintain the dimerized state of TcpP as a transcriptional regulator (Fig. 8B).

This model (Fig. 8) also suggests why the increased percentage of reduced TcpP in the presence of TC could only be observed at the beginning of arabinose induction (Fig. 7A). At the beginning of induction, there was lower level of TcpP. In the presence of TC, VcDsbA is only partially in an oxidized state. Some newly synthesized TcpP will escape VcDsbA oxidization and remain in a reduced form (Fig. 8B). However, reduced TcpP will quickly interact with oxidized TcpP (intramolecular S—S) to form an intermolecular disulphide bond between the 207th cysteine residues in two TcpP molecules, which is essential to TcpP homodimerization (Yang *et al*., 2013). The free cysteine (C218) would also be oxidized quickly by VcDsbA (Fig. 8B). As TcpP expression increases, so does TcpP oxidation, eventually becoming independent of TC concentrations.

At high concentrations, bile salts act as effective protein-unfolding agents and instigators of disulphide stress *in vivo* (Cremers *et al*., 2014). In the periplasm, a relatively oxidized chamber of the bacteria (Messens *et al*., 2007), bile salts inhibit disulphide bond formation by interfering with the redox potential of Dsb proteins, while in the cytoplasm, which is more reduced (Gilbert, 1990; Holmgren and Fagerstedt, 1982), bile salts instigate disulphide stress by changing the GSH/GSSG redox potential through unknown redox components (Cremers *et al*., 2014).

The Dsb redox system plays a pivotal role in the virulence of many pathogens. *Francisella tularensis* DsbA is required for infection of macrophages and mice (Qin *et al*., 2009) and two essential virulence factors are the substrates of FtDsbA (Ren *et al*., 2014). The DsbA enzyme from *Pseudomonas aeruginosa* (PaDsbA) is required for the expression of elastase, exotoxin A, protease IV, and is also required for the formation of a functional type III secretion system (Braun *et al*., 2001; Ha *et al*., 2003). Bile salts can induce virulence gene expression of *Vibrio parahaemolyticus* via the VtrA/VtrB regulatory system (Gotoh *et al*., 2010), although the mechanism is unknown.

In this study, we provide evidence that TC binds some Dsb proteins from *V. cholerae* and *E. coli* and affects the activity of these proteins. *V. cholerae* takes advantage of how TC affects VcDsbA activity to induce virulence gene expression. It is likely that bile salts will also affect other pathogens. Our work suggests that molecules able to alter DsbA function may be efficacious antimicrobials.

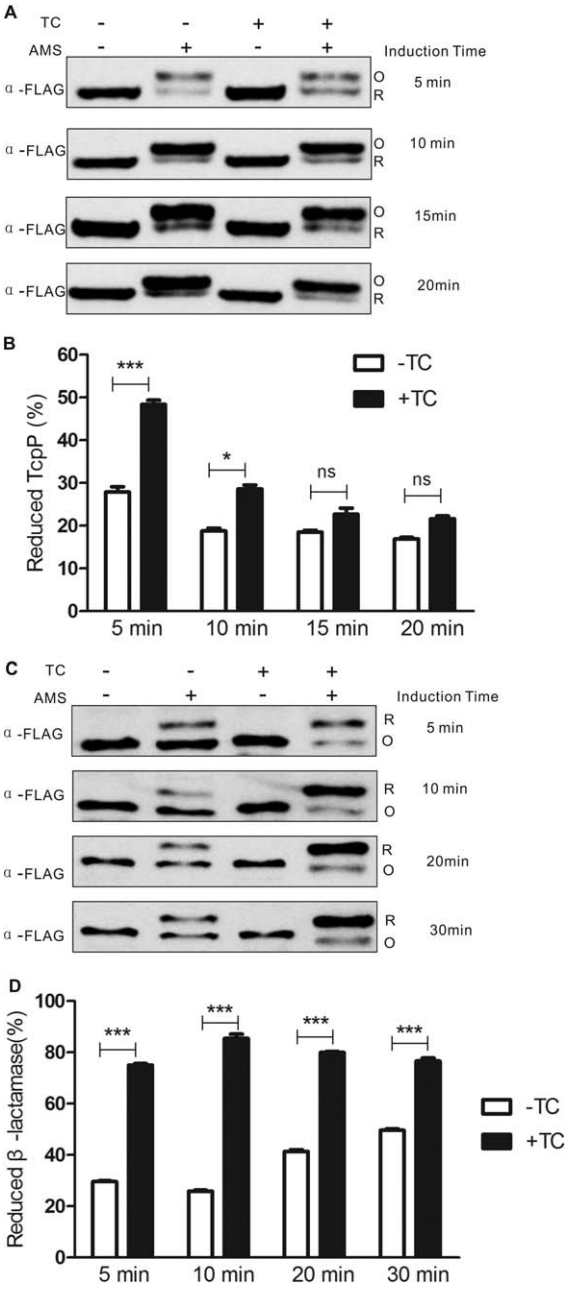

Fig. 7. Redox status assays of VcDsbA substrates.
A. TcpP redox status assay *in vivo*. TcpP-cFLAG was overexpressed in *V. cholerae* Δ*tcpP* grown in LB medium. To label free thiol groups irreversibly, 100 mM iodoacetamide was added directly to the living cells. After TCA precipitation and extensive washing, oxidized thiol groups were reduced by addition of 50 mM DTT in denaturing buffer. These reduced cysteines were then alkylated by addition of 10 mM AMS (+) or not (−). Samples were mixed with non-reducing SDS-sample buffer and loaded onto 12.5% SDS-polyacrylamide gels. TcpP was detected by Western blot analysis of the FLAG-tagged proteins.
B. Quantification of band intensities of TcpP from blot shown in panel A was performed using ImageJ software. Graph represents percentages of reduced TcpP. Data shown are averages of three independent experiments. ***, $P < 0.0001$. *, $P < 0.05$.
C. β-lactamase redox status assay by AMS trapping in the presence or absence of TC. β-lactamase containing four consecutive cysteine residues and the FLAG tag in the C-terminus was overexpressed under the control of the P_{BAD} promoter in *V. cholerae* Δ*dsbC* grown in LB medium in the presence or absence of TC. After different time of arabinose induction as indicated, proteins were precipitated by 10% TCA and free thiol groups were labelled by AMS. Different forms of β-lactamase were separated by SDS-PAGE and detected by Western blot analysis of the FLAG-tagged proteins.
D. Quantification of band intensities from blot shown in panel C was performed using ImageJ software. Graph represents percentages of reduced β-lactamase. Data shown are averages of three independent experiments. ***, $P < 0.0001$. Blot shown is representative of at least three separate experiments.

Experimental procedures

Bacterial strains, plasmids and growth conditions

Strains, plasmids and oligonucleotides used in this study are summarized in Supporting Information Table S1. All *V. cholerae* strains used in this study were derived from E1 Tor C6706 (Joelsson *et al.*, 2006) and were propagated in LB media containing appropriate antibiotics at 37°C. Transcriptional *lux* reporters of *toxT* promoter regions in the pBBR-lux vector (Liu *et al.*, 2011) have been described previously (Yang *et al.*, 2013). Plasmids for overexpressing TcpP or mutants were either described previously (Yang *et al.*, 2013) or constructed by cloning the PCR-amplified coding regions into pBAD24 (Guzman *et al.*, 1995) or pACYC117 (Chang and Cohen, 1978). In-frame deletion strains used in this study were described in previous publications (Liu *et al.*, 2011; Yang *et al.*, 2013). TcpP truncation and cysteine mutations were constructed by using overlap extension PCR (Higuchi *et al.*, 1988).

DsbA cloning, expression and purification

The *V. cholerae dsbA and dsbC* genes (accession codes NP_229693.1 and NP_232048.1) were amplified from *V. cholerae* genomic DNA. *EcdsbA*, *EcdsbC* and *EctrxA* (accession codes NP_418297.1, NP_417369.1 and NP_418228.2) were amplified from *E. coli* genomic DNA. *MtdsbA* (accession code NP_217485.1) was amplified from *M. tuberculosis* genomic DNA, a gift from Dr. Xiangmei Zhou. Primers (Supporting Information Table S1) for each gene were used and compatible with ligation-independent cloning. The PCR product without the signal peptide sequence was then inserted into a modified pET-28a vector encoding an N-terminal His_6 tag. VcDsbA cysteine mutations were constructed using overlap extension PCR (Higuchi *et al.*, 1988). *E. coli* BL21(DE3)/pLysS cells were transformed with the plasmid containing the target gene and transformed cells were used for protein expression by autoinduction (Thompson *et al.*, 1994). Proteins were expressed and purified on nickel columns according to the manufacturer's instructions (Qiagen).

Measurement of virulence gene expression and virulence factor production

Overnight cultures of *V. cholerae* strains containing virulence promoter *luxCDABE* transcriptional fusions were

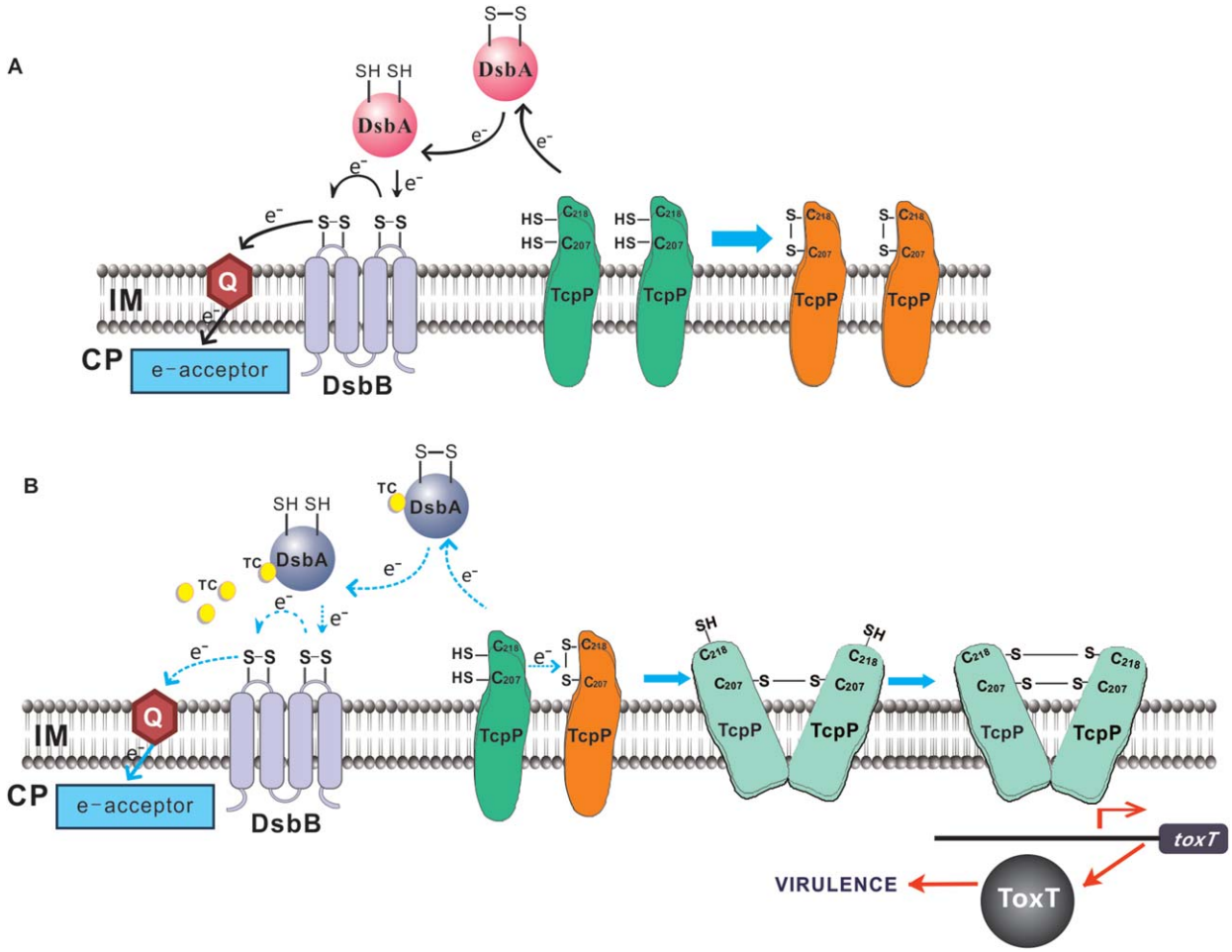

Fig. 8. Working model. TC interference with VcDsbA redox reactions results in TcpP intermolecular disulphide bond formation. In the absence of TC, VcDsbA is functional and quickly oxidizes TcpP to form inhibitory intramolecular disulphide bonds (A); in the presence of TC, VcDsbA will be only partially in the oxidized form, and a portion of newly synthesized TcpP may escape from VcDsbA oxidation to form intermolecular disulphide bonds, which helps to maintain the dimerized state of TcpP as functional transcriptional regulator (B).

subcultured at a dilution of 1:100 in LB with or without 1 mM of TC as indicated and grown anaerobically until $OD_{600} \approx 0.2$. Luminescence was measured using a Bio-Tek Synergy HT spectrophotometer and normalized for growth against OD_{600}. Luminescence expression was reported as light units/OD_{600}. ToxT production was measured by Western blot analysis using an anti-ToxT polyclonal antibody (Zhu *et al.*, 2002).

Insulin reduction assay

The protein disulphide reductase activity of VcDsbA was measured *in vitro* using the insulin-reduction assay in the presence of DTT (Holmgren, 1979). Each DsbA protein (5 or 10 μM) was mixed with buffer consisting of 100 mM sodium phosphate, 2 mM EDTA, 0.33 mM DTT with or without different concentrations of TC as indicated. Insulin (0.131 mM) was added immediately before measurements were made. The reduction of insulin by DTT was monitored spectrophotometrically at 650 nm.

Bacterial two-hybrid system to determine TcpP–TcpP interaction

To analyze the dimerization of TcpP, β-galactosidase measurements were performed as described previously (Yang *et al.*, 2013). Briefly, overnight cultures of *E. coli* BTH101 or BTH101Δ*dsbA* mutants containing both pUT-18C-fusion and pKT25-fusion constructs (Karimova *et al.*, 1998) were subcultured at a dilution of 1:100 in LB medium containing 0.5 mM isopropyl-β-D-1-thiogalactopyranoside and incubated without shaking at 30°C for 8 h. About 1 mM of TC and different concentrations of cysteine were added as indicated. Cultures were then assayed for β-galactosidase activity (Karimova *et al.*, 1998).

TcpP-oxidation assay

TcpP-oxidation assays were performed by using membrane preparations (Poole, 1993) obtained either from *E. coli strain* Δ*dsbA* producing $TcpP_{C19S/C124S}$ or $TcpP_{C19S/C124S/}$

$_{C207S/C218S}$ with a FLAG-tag fusion in the C-terminal grown in LB broth supplemented with 0.2% (wt/vol) of arabinose and appropriate antibiotics. Membrane protein concentrations were determined using the quick-start Bradford dye reagent (Bio-Rad) with bovine serum albumin (BSA) as a standard. Different volumes of 50 μM oxidized VcDsbA in 50 mM MES, 50 mM NaCl, 2 mM EDTA pH 5.5 with or without different concentrations of TC as indicated were added to the same buffer containing 1 mg/mL TcpP membrane suspension which was reduced by 20 mM DTT and DTT was removed later by PD-10 column (GE). The mixture was then incubated at 30°C for 30 min. Proteins were precipitated in 10% (vol/vol) trichloroacetic acid (TCA) at 4°C overnight. Precipitated proteins were washed three times with cold acetone, suspended in a buffered solution containing 100 mM Tris-HCl pH 7.5 and 1% (wt/vol) SDS. Different forms of TcpP were separated by non-reducing SDS-PAGE and detected by monoclonal anti-FLAG antibody (Sigma-Aldrich).

Isothermal titration calorimetry

Evaluation of affinity and thermodynamics of binding between bile salts and DsbA proteins were assessed by isothermal titration calorimetry (ITC) using a VP-ITC instrument (MicroCal^{TM}, GE Healthcare). The sample cell was loaded with 1.5 mL of purified protein at 200 μM concentrations in 50 mM MES, pH 5.5, 150 mM NaCl, 2 mM EDTA (for VcDsbA) or PBS (for EcDsbA, TrxA, MtDsbA, EcDsbC and VcDsbC). The syringe was filled with TC in the same buffer as that was used to dilute the proteins at a concentration of 4 mM. Titrations were conducted at 25°C using 25 consecutive injections of 10 μL, each delayed by 300 s with a stirring speed of 307 rpm. As a control for background noise, titration of TC into a solution containing the buffer only was performed. The association constant ($K_a = 1/K_d$), free energy (ΔG) and enthalpy change (ΔH) and entropy change (ΔS) were calculated by fitting the data to a single-site binding model using the MicroCal Origin software (Origin 7.0 SR4 version7.0552β). Parameters reported include the mean ± S.D. of three replicates. The calculated *c*-value for these measurements was 12.

Redox potential determination

VcDsbA redox potential assay was performed essentially as described (Walden *et al.*, 2012). Oxidized VcDsbA (2 μM) was incubated in fully degassed buffer consisting of 100 mM sodium phosphate, 1 mM EDTA pH 7.0 containing 1 mM oxidized glutathione (GSSG; Sigma-Aldrich, The United States) and a range of reduced glutathione (GSH) concentrations (0.1–2.0 mM) for 24 h at room temperature. TC was at a concentration of 20 μM. After incubation, the reactions were stopped with 10% TCA and the precipitated protein pellets were collected by centrifugation at 16,000*g* for 30 min at 4°C. The pellets were washed with 100% ice-cold acetone and dissolved in a buffer consisting of 50 mM Tris–HCl pH 7.0, 1% SDS, 10 mM 4-acetamide-4′-maleimidylstilbene-2,2′- isulphonate (AMS) to label the free thiols. Separation of the reduced and oxidized forms was performed on 12% SDS-polyacrylamide gels under denaturing conditions. Gels were stained with Coomassie Brilliant Blue and scanned. The relative intensity of the reduced and oxidized forms was analyzed using ImageJ (http://rsbweb.nih.gov/). The fraction of the reduced protein was plotted against the ratio $[GSH]^2/[GSSG]$.

In vivo *redox state determination*

The *in vivo* redox state of VcDsbA or beta-lactamase was assessed using AMS trapping experiments as (Denoncin *et al.*, 2013). Bacteria harbouring a plasmid encoding VcDsbA, TcpP or β-lactamase under the control of an arabinose inducible promoter were cultured at 37°C with 200 rpm shaking in LB medium with or without 1 mM of TC as indicated until they reached an optical density at 600 nm (OD_{600}) of 0.5. Arabinose (0.1%) was added to the culture and bacteria were kept cultured in the same conditions until 10% (vol/vol) trichloroacetic acid (TCA) was added. Proteins were precipitated overnight at 4°C. Precipitated proteins were washed three times with cold acetone, suspended in a buffered solution containing 100 mM Tris-HCl pH 7.5 and 1% (wt/vol) SDS, with or without 10 mg/mL AMS, and incubated in the dark at 30°C for 30 min followed by 37°C for 10 min. AMS alkylation was stopped by the addition of SDS loading buffer [2% (wt/vol) SDS, 50 mM Tris, 10% (vol/vol) Glycerol, 142 mM 2-mercaptoethanol]. Proteins were separated by SDS-PAGE, and immunoblot analyses were performed.

VcDsbA oxidized by VcDsbB present in membranes

Vibrio cholerae strain Δ*dsbA* harbouring a plasmid encoding VcDsbB under the control of an arabinose inducible promoter was cultured at 37°C with 200 rpm shaking in LB medium until they reached an optical density at 600 nm (OD600) of 0.5. Arabinose (0.2%) was added and bacteria were kept cultured in the same conditions for another 12 h. Membranes were prepared according to (Kadokura and Beckwith, 2009). Purified DsbA was reduced by incubation in 20 mM DTT for 10 min at 4°C. DTT was then removed by gel filtration on PD-10 Sephadex columns (GE). Reduced DsbA was stored at −80°C in the presence of 0.1 mM EDTA, pH 8.0. VcDsbB membrane suspension (1 mg/mL) was mixed with 10 mM ubiquinone 1 (UQ1) with or without different concentrations of TC as indicated in PBS. Reactions were started right after 2 μM of reduced

VcDsbA was added. TCA (10%) was added at different time points to stop the reaction and protein was precipitated at 4°C overnight. Precipitated proteins were treated with AMS as described. Negative controls were the membrane of *V. cholerae* Δ*dsbA/dsbB*.

PDI detector construction

Bla with mutations of S81C (TCA to TGT) and T108C (ACT to TGT) was obtained by overlap extension PCR and substituted the wild type Bla cassette in pACYC177 (Chang and Cohen, 1978). The plasmid was transferred into *V. cholerae* by electroporation (Liu *et al.*, 2011). VcdsbC or VcdsbA or the mutants were constructed by cloning the PCR-amplified coding regions into pBBRMCS3 (Kovach *et al.*, 1995) and transferred into *V. cholerae* PDI detector strains by conjugation.

Spot titres for ampicillin resistance, cadmium resistance

Spot titres for ampicillin resistance were performed to test β-lactamase activity *in vivo*. Cadmium resistance was performed to quantify the relative disulphide oxidase activity of the strains *in vivo*. Briefly, strains were grown overnight in LB and diluted 1:100 into fresh LB media with appropriate antibiotics containing 1 mM of TC or not as indicated. Strains were grown to mid-logarithmic phase at 37°C and serially diluted into phosphate buffer salt (PBS). A 5 μL aliquot of each dilution was plated onto LB plates with an ampicillin or cadmium gradient. Cells were grown at 37°C overnight. All spot titres were performed at least in triplicates.

Melting-temperature measurements

Thermal unfolding was performed by measuring CD spectra on a Chira scan spectropolarimeter (Applied Photophysics Ltd., Leatherhead). Unfolding of reduced or oxidized VcDsbA was induced by increasing the temperature from 25°C to 105°C with a heating rate of 1°C/min. For both proteins, the conditions used were 10 μM protein in 100 mM sodium phosphate and 1 mM EDTA pH 7.0 with or without 100 μM TC as indicated. The redox state of the oxidized protein was verified by Ellman's assay. Thermal unfolding measurements of reduced VcDsbA were performed in the presence of 0.75 mM DTT. Unfolding was monitored by circular-dichroism measurements and was analyzed using Prodata viewer (Applied Photophysics Ltd., Leatherhead). Three replicates were measured for each of the reduced and oxidized enzymes.

CD measurements

Reduced or oxidized VcDsbA (10 μM) was prepared in 100 mM sodium phosphate and 1 mM EDTA pH 7.0 with or without 100 μM TC and incubated for 1 h at 30°C. Far-UV–CD spectra were recorded at the indicated temperature in a Chira scan spectropolarimeter (Applied Photophysics Ltd., Leatherhead).

Differential thiol trapping of TcpP *in vivo*

The thiol/disulphide state of the periplasmic cysteines of TcpP was monitored *in vivo* by differential thiol trapping (Tetsch *et al.*, 2011). The procedure was modified as follows: *V. cholerae* Δ*tcpPH* strain carrying the plasmid pBAD–TcpP–cFLAG were grown in LB medium with or without 1 mM of TC to an OD_{600} of around 0.5. Subsequently, overproduction of the TcpP–cFLAG derivatives was induced by addition of 0.1% (w/v) arabinose for the time as indicated. About 100 mM iodoacetamide was added, and incubated at 37°C with 130 rpm agitation for 1 h. This first alkylation procedure irreversibly modified all free thiol groups. Subsequently, cells were lysed in ice-cold 10% (w/v) TCA and stored on ice for at least 6 h or at 4°C overnight. The TCA treated cells were centrifuged (16,000*g*, 4°C, 15 min), and the resulting pellet was washed twice with 500 μL of ice-cold acetone. The supernatant was removed and the pellet was resuspended in 900 μL of a buffer solution [100 mM Tris-HCl (pH 7.5), 1% (w/v) SDS] supplemented with 50 mM DTT to reduce disulphide bonds. After one hour of incubation in the dark (37°C, gentle agitation at 1,300 rpm), 100 μL ice-cold 100% (w/v) TCA was added, and the sample was stored on ice for at least 6 h. After centrifugation, the resulting pellet was again washed twice with 500 μL of ice-cold acetone. Finally, the pellet was resuspended in 50 μL of Tris-SDS buffer, with or without 10 mM of AMS, and incubated at 30°C for 30 min followed by 37°C for 10 min. AMS alkylation was stopped by the addition of SDS loading buffer. Proteins were separated by SDS-PAGE, and immunoblot analyses were performed as described above. TcpP was detected by monoclonal anti-FLAG antibody (Sigma-Aldrich).

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interpretation or the decision to submit the work for publication.

Author contributions

X. Y., M. S., F. T., C. W. and X. W. performed research (the acquisition, analysis or interpretation of the data); W. F. contributed new reagents/analytic tools; G. R., H. S. and M. Y. analyzed data; G. R., H. S. and M. Y. designed research and wrote the manuscript.

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