Contact-independent killing mediated by a T6SS effector with intrinsic cell-entry properties

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Bacterial type VI secretion systems (T6SSs) inject toxic effectors into adjacent eukaryotic and prokaryotic cells. It is generally thought that this process requires physical contact between the two cells. Here, we provide evidence of contact-independent killing by a T6SS-secreted effector. We show that the pathogen *Yersinia pseudotuberculosis* uses a T6SS (T6SS-3) to secrete a nuclease effector that kills other bacteria in vitro and facilitates gut colonization in mice. The effector (Tce1) is a small protein that acts as a Ca$^{2+}$- and Mg$^{2+}$-dependent DNase, and its toxicity is inhibited by a cognate immunity protein, Tci1. As expected, T6SS-3 mediates canonical, contact-dependent killing by directly injecting Tce1 into adjacent cells. In addition, T6SS-3 also mediates killing of neighboring cells in the absence of cell-to-cell contact, by secreting Tce1 into the extracellular milieu. Efficient contact-independent entry of Tce1 into target cells requires proteins OmpF and BtuB in the outer membrane of target cells. The discovery of a contact-independent, long-range T6SS toxin delivery provides a new perspective for understanding the physiological roles of T6SS in competition. However, the mechanisms mediating contact-independent uptake of Tce1 by target cells remain unclear.
To survive in complex microbial communities such as the intestinal microbiota, bacteria have evolved various molecular weapons to compete with other species. The classic examples of these weapons are diffusible antimicrobials such as broad-spectrum antibiotics and strain-specific bacteriocins (including microcins, bacteriocins of less than 10 kDa produced through the ribosomal pathway), which can exert long-range inhibitory effects on target cells. Some gram-negative bacteria use contact-dependent growth inhibition (CDI), composed by CdiB translocators and CdiA toxins, to compete with other bacteria. Similar to bacteriocins and microcins, CDI requires specific outer membrane (OM) receptors on susceptible bacteria for translocation of CdiA toxins into the target cell. The type VI secretion system (T6SS) is another contact-dependent weapon in bacteria. Similar to bacteriocins and microcins, CDI requires a cognate immunity protein, which protects the producing cells from self-toxicity. All contact-dependent T6SS antibacterial weapons characterized to date do not require specific receptors in target cells for delivery of effectors or recognition of prey cells. Recently, Si and colleagues reported a contact-independent role of T6SS in metal acquisition through secretion of metal-scavenging effectors into host cells. T6SS is primarily considered an antibacterial weapon to compete against rival bacteria in polymicrobial environments. The antibacterial function of T6SSs relies on injection of antibacterial effectors that target conserved, essential features of the bacterial cell. Each antibacterial effector is co-expressed with a cognate immunity protein, which protects the producing cells from self-toxicity. All contact-dependent T6SS antibacterial weapons characterized to date do not require specific receptors in target cells for delivery of effectors or recognition of prey cells. Recently, Si and colleagues reported a contact-independent role of T6SS in metal acquisition through secretion of metal-scavenging effectors into host cells. T6SS is primarily considered an antibacterial weapon to compete against rival bacteria in polymicrobial environments.

**Results**

**Identification of novel T6SS effectors.** To identify novel T6SS effectors, we searched the Yptb YPIII genome for genes containing the Proline-Alanine-Alanine-Arginine (PAAR) domain, a conserved effector-targeting domain that is linked or adjacent to numerous known T6SS effectors. A gene locus encoding multiple hypothetical T6SS effector-immunity pairs was identified (YPK_0952-0958, Fig. 1a). Both the first and last open-reading frame (ORF) of this locus contain PAAR domains. The first ORF, YPK_0952, contains a typical PAAR domain at its N-terminus and an S-type pyocin domain at its C-terminus. When VSVG-tagged YPK_0952 was produced in YPIII, the secreted protein was readily detected in the supernatant. However, YPK_0952 secretion was abrogated in the ΔclpV mutant, in which all four essential ATPase genes in the four sets of T6SSs were deleted, strongly suggesting that YPK_0952 is a T6SS effector. The secretion of YPK_0952 was dramatically diminished with deletion of clpV3, but not with deletion of clpV1, clpV2, or clpV4, further indicating that YPK_0952 is a T6SS effector mainly associated with T6SS-3 (Supplementary Fig. 1a). Similarly, we showed that YPK_0954, which does not contain a PAAR domain but is located downstream of YPK_0952, is also a T6SS-3 effector (Fig. 1b and Supplementary Fig. 1b).

To confirm the toxic activity of YPK_0954, we performed toxicity assays in *Escherichia coli*. Expression of YPK_0954, a 67-amino acid (aa) protein in *E. coli* results in significant growth inhibition. This growth inhibition was relieved by co-expression of the immediately downstream gene YPK_0955, which encodes a protein containing the colicin Immunom domain in the ypk_0954-ypk_0955 bicistron (Fig. 1c). This result suggests that YPK_0955 is the cognate immunity protein for YPK_0954. We renamed YPK_0954 as T6SS contact-independent antibacterial effector 1 (Tce1) and the immunity protein YPK_0955 as T6SS contact-independent antibacterial immunity 1 (Tci1) for reasons described below.

To assess whether the immunity results from direct protein-protein interaction, we performed glutathione S-transferase (GST) pull-down and bacterial two-hybrid assays, and the results showed specific interactions between Tce1 and Tci1 (Fig. 1d, e). These results indicate that Tce1 is a T6SS-3 secreted antibacterial effector and that its toxicity is neutralized by the Tci1 immunity protein.

**Tce1 exhibits Ca$^{2+}$, Mg$^{2+}$-dependent DNase activity.** Having demonstrated that Tce1 is a T6SS effector, we sought to investigate its biochemical activity. No predictable functional domain could be identified in Tce1 using the BLASTP search or other bioinformatics tools. However, further analysis using HHpred revealed similarity of Tce1 with the DNA-binding proteins BldC in *Streptomyces coelicolor* and Ceda in *E. coli*, implying its potential role as a nuclease toxin. Incubation of chromatography-purified Tce1 (Supplementary Fig. 1c) with λ-DNA in the same reaction buffer as DNase I led to dramatic DNA degradation in a pattern similar to DNase I (Fig. 1f). The DNase activity of Tce1 critically relies on the co-existence of Ca$^{2+}$ and Mg$^{2+}$, and addition of excess EDTA inhibited the activity of both Tce1 and DNase I (Fig. 1g and Supplementary Fig. 1d). The circular plasmid pUC19 was also assayed as a substrate (Supplementary Fig. 1e, f), further indicating that Tce1 is an endonuclease. Based on optical density measurements and gel electrophoresis, Tce1 had lost toxicity to *E. coli* (Tce1$^{S8A/A16E}$) from approximately 400 candidates mutated at the DNase active site (Supplementary Fig. 1c). The purified mutant protein (Supplementary Fig. 1c) failed to cleave DNA as the Tce1 wild-type (WT) protein (Fig. 1h and Supplementary Fig. 1g), clearly demonstrating that the DNase activity of Tce1 is not due to contamination. However, Tce1 did not display detectable RNase activity in vitro (Supplementary Fig. 1h, i). Therefore, Tce1 is a Ca$^{2+}$, Mg$^{2+}$-dependent endonuclease that cleaves DNA but not RNA. Consistent with its role as an immunity protein for Tce1, addition of Tci1 to the reaction mixture effectively diminished the DNase activity of Tce1 (Supplementary Fig. 1j).

The DNase activity of YPK_0954 was further confirmed in vivo using the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay and DAPI staining. Although most *E. coli* cells expressing Tce1 exhibited positive TUNEL signals, indicative of DNA fragmentation, *E. coli* cells expressing Tce1$^{S8A/A16E}$ and co-expressing Tce1-Tci1, remained unlabeled, similar to the vector-only control (Fig. 1i). Furthermore, more than 80% of the *E. coli* cells expressing Tce1 lost DAPI staining after 4 h induction with IPTG. However, minimal loss of DAPI staining was observed in cells harboring empty vector, expressing Tce1$^{S8A/A16E}$, or co-expressing Tce1-Tci1 (Fig. 1j and Supplementary Fig. 2). These results establish that Tce1 is an actual DNase.
Tce1 mediates contact-independent T6SS killing. To assess the contribution of the Tce1-Tci1 effector-immunity pair to bacterial antagonism, we performed growth competition assays using labeled derivatives of Yptb co-cultured under conditions promoting cell contact. Notably, the Tce1-Tci1 effector-immunity pair apparently underwent a duplication event based on the identification of a highly homologous gene pair yp0_2801-yp0_2802 in the Yptb genome through a homology search (Supplementary Fig. 3). To simplify the analysis, we generated a deletion mutant lacking yp0_2801-yp0_2802 as the WT for subsequent experiments. The WT donor exhibited a 3-fold growth advantage in competition with the Δtce1Δtci1 recipient. This growth advantage was abrogated by deletion of tce1 from the donor, or by expression of tci1 in the recipient. The Tce1-mediated growth advantage requires a functional T6SS-3, as deletion of clpV3 (ΔclpV3*, clpV3 deleted in the Δypk_2801Δypk_2802 background) completely abolished the growth advantage (Fig. 2a).

Unexpectedly, a strong competitive advantage dependent on Tce1 was also observed when cells were grown in liquid medium. As shown in Fig. 2a, donor strains possessing Tce1 and a...
functional T6SS-3 exhibited significantly increased fitness in competition with Δtce1Δtci1 recipients, and expression of tci1 in the Δtce1Δtci1 recipient rescued its competitive fitness (Fig. 2a). Similar results were obtained when the assay was repeated with a cell-impermeable membrane separating the donor and recipient cells on the surface of solid medium (Fig. 2b). Thus, T6SS-3 confers bacteria a contact-independent competitive advantage due to secretion of an antibacterial effector into the extracellular medium, which is distinct from the canonical contact-dependent T6SS mechanism that acts as a conduit to deliver effectors across the envelope of recipient cells25–27.

As a nuclease toxin, we assumed that Tce1 secreted into the extracellular medium must subsequently enter recipient cells to access its DNA target. To verify this prediction, we performed a
teriocins and microcins are known to recognize specific targets. By contrast, addition of AF488-conjugated Tce1, a canonical *Pseudomonas aeruginosa* T6SS toxin that requires the T6SS needle to puncture the target cell for translocation, did not yield fluorescent bacteria (Fig. 2c and Supplementary Fig. 4a). The contact-independent killing activity of Tce1 was also verified by examining its toxicity to target cells in liquid medium. While addition of purified Tce1 protein to the liquid medium had little effect on WT survival, it greatly reduced the survival rate of the ∆tce1Δtci1 mutant, which lacks the immunity protein (Fig. 2d and Supplementary Fig. 5). Conversely, consistent with a previous report, the canonical T6SS toxin Tse1 exhibited no toxic effect on both bacterial strains under the same conditions (Fig. 2d). These results suggest that, unlike canonical T6SS effectors, the Tce1 effector possesses an intrinsic cell-entry mechanism. Once released into the extracellular medium, this cell-entry mechanism allows Tce1 to recognize and enter target cells independent of the T6SS needle.

Through the AF488-based protein importation assay, we found that its cell-entry mechanism also allows Tce1 to specifically enter the cytosol of *E. coli* and *Salmonella* Typhimurium, but not that of *Salmonella enteritidis*, *Acinetobacter baumannii*, or *Corynebacterium glutamicum* (Fig. 2e and Supplementary Fig. 4b). In line with these results, exogenous addition of recombinant Tce1 protein to the liquid medium substantially reduced the survival rates of *E. coli* and *S. Typhimurium*, but not of *S. enteritidis*, *C. glutamicum*, or *A. baumannii* (Fig. 2f). These findings motivated us to further investigate whether Tce1 participates in contact-independent inter-species antagonism. As expected, Tce1 significantly contributed to the fitness of *Yptb* against *E. coli* and *S. Typhimurium* during contact-independent competition assays performed in liquid medium (Fig. 2g).

Together, these results demonstrate that *Yptb* T6SS-3 follows a non-canonical contact-independent killing mechanism mediated by secretion of Tce1, a unique antibacterial effector with an intrinsic cell-entry mechanism.

**Tce1 interacts with the OM receptors BtuB and OmpF.** Bacteriocins and microcins are known to recognize specific receptors on sensitive cells to traverse the cell envelope. Thus, we hypothesized that Tce1 may also interact with membrane receptors to enter target cells. To identify such putative receptors, we performed GST pull-down assays using GST-Tce1-coated beads against total cell lysates of *Yptb* WT cells. Proteins specifically retained by GST-Tce1 were detected using silver staining after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. 3a). Mass spectrometric analysis identified several potential Tce1 partners: the 80 kDa band as BtuB, a TonB-dependent OM receptor (YPK_0782; Supplementary Fig. 6); the 40 kDa band as the OmpF porin (YPK_2649; Supplementary Fig. 7); the band around 50 kDa as TolB (YPK_2956), the periplasmic component of the TolABQR translocation machinery for group A colicin importation; the 38 kDa band as an OmpA domain transmembrane region-containing protein (YPK_2630); and the 90 kDa band as AcnB (aconitate hydratase 2, YPK_3487). As the involvement of BtuB, OmpF, and TolB in bacteriocin and microcin importation has been well established, we focused further investigation on BtuB, OmpF, and TolB. The specific interactions of Tce1 with BtuB, OmpF, and TolB were further supported by bacterial two-hybrid (Fig. 3b) and in vitro binding assays (Fig. 3c–e).

**Potential roles of BtuB and OmpF in Tce1 cell entry.** To test potential roles of BtuB and OmpF in Tce1 cell entry, we performed a fluorescence-based assay using Alexa Fluor 488-conjugated Tce1 to probe its import in vivo. Although addition of Tce1-AF488 to WT *Yptb* and ∆btuB or ∆ompF mutants yielded fluorescent bacteria, the ∆btuBΔompF mutant was not labeled. Labeling of ∆btuBΔompF was weakly but substantially restored through complementation of ompF, and strongly restored through complementation of btuB (Fig. 4a and Supplementary Fig. 4c). In addition, we repeated the experiment using GFP labeling instead of AF488. Although exogenous GFP-Tce1 protein could not enter into the cytoplasm of target cells, it specifically labeled bacterial cells expressing ompF or btuB on the cell surface. In contrast, ∆btuBΔompF was not labeled with GFP-Tce1 protein (Fig. 4b and Supplementary Fig. 4d). The BtuB/OmpF-dependent entry of Tce1 into cytosol was further confirmed based on a cell fractionation experiment. As shown in Supplementary Fig. 8, Tce1 was detected in the cytosol of WT *Yptb* but not the ∆btuBΔompF mutant. However, complementation of btuB partially restored cytosolic Tce1 to the WT level.

These observations indicate that BtuB or OmpF are required for efficient uptake of Tce1 by target cells. Thus, *Yptb* with mutated BtuB or OmpF can be expected to show natural resistance to Tce1, even when the immunity protein Tci1 is not present. We tested this hypothesis by treating various mutants with different concentrations of purified Tce1 in M9 medium. As expected, deletion of btuB or ompF somewhat reduced the sensitivity of the ∆tce1Δtci1 mutant to exogenously supplied Tce1 protein, while the ∆tce1Δtci1∆btuBΔompF quadruple mutant was not sensitive to Tce1 protein. However, the sensitivity of ∆tce1Δtci1∆btuBΔompF was weakly restored through complementation of ompF, and strongly restored through complementation of btuB (Fig. 4c and Supplementary Fig. 5a–c). This also explains why *E. coli* and *S. Typhimurium*, which contain highly similar BtuB and OmpF homologs, are sensitive to Tce1 treatment, but *S. enteritidis*, *C. glutamicum*, and *A. baumannii*, which do not contain such highly similar BtuB and OmpF homologs, are immune to Tce1 treatment (Supplementary Figs. 6 and 7).

Consistent with its crucial role in facilitating bacteriocin transfer across the OM, the tolB deletion mutant was also not sensitive to Tce1 treatment, and complementation of tolB restored the sensitivity to the WT level (Supplementary Fig. 9a). Moreover, while Tce1-AF488 labeled the whole cell of WT *Yptb* and the ∆tolB(tolB) complemented strain, it only weakly labeled the ∆tolB mutant on the cell surface (Supplementary Fig. 9b, c), further supporting that TolB plays a role in facilitating Tce1 translocation across the OM.

To further investigate whether BtuB and OmpF are involved in Tce1-mediated contact-independent T6SS killing, intra-species competition assays were performed in liquid medium. Although the WT strain strongly inhibited the growth of ∆tce1Δtci1, it failed to inhibit the growth of the ∆tce1Δtci1∆btuBΔompF mutant. However, the reduced sensitivity in the ∆tce1Δtci1∆btuBΔompF mutant was substantially restored by complementation of ompF or btuB (Fig. 4d). Similar results were obtained when the assay was repeated with a cell-impermeable membrane separating the donor and recipient cells on the surface of solid medium (Supplementary Fig. 10). Similarly, the *E. coli* ∆btuBΔompF mutant was clearly more tolerant to WT *Yptb* attack than the WT *E. coli* in liquid medium (Fig. 4e), further supporting that both BtuB and OmpF are required for Tce1-mediated contact-independent T6SS killing.

We also examined the roles of BtuB and OmpF in Tce1-mediated contact-dependent T6SS killing on the surface of solid medium. Unexpectedly, the WT *Yptb* caused stronger inhibition of the growth of the ∆tce1Δtci1∆btuBΔompF mutant compared with the ∆tce1Δtci1 mutant (Supplementary Fig. 11a). A potential explanation for this apparent discrepancy is that the membrane of the ∆tce1Δtci1∆btuBΔompF mutant is vulnerable to T6SS attack. Consistent with this possibility, we found that WT *Yptb* exhibited stronger inhibition of...
the growth of the E. coli ΔbtuBΔompF mutant compared to WT E. coli during contact-dependent competition (Supplementary Fig. 11b). Collectively, these results indicate that BtuB and OmpF are important for contact-independent, but not for contact-dependent, entry of Tce1.

The Tce1-mediated T6SS killing pathway facilitates gut colonization. Some enteric pathogens have been reported to use T6SS to kill symbionts and become established in the mammalian gut. To investigate the role of Tce1 in facilitating Yptb colonization of the gastrointestinal tract, streptomycin-treated and untreated mice were orally infected with equivalent doses (10^9 colony-forming units, CFUs) of WT Yptb or the Δtce1 and ΔclpV3* mutant. The degree of colonization of the cecum and small intestine of infected mice was determined at 24 and 48 h post-infection (Fig. 5a, b and Supplementary Fig. 12a, b). Without streptomycin treatment, the CFU level of Δtce1 and ΔclpV3* was significantly lower compared with the WT in both organs. These results demonstrate that the Tce1-mediated T6SS killing pathway is required for colonization of the mouse gut, as it allows for outcompeting of gut commensals. This conclusion was supported by the finding that expression of the tce1 and T6SS-3 genes was strongly induced during mouse infection compared with growth in YLB medium (Supplementary Fig. 12c). However, pre-treatment with antibiotics greatly reduced the observed difference between the WT and Δtce1 in both organs, and between the WT and ΔclpV3* in the small intestine (Fig. 5a, b), indicating that the Tce1-mediated T6SS antibacterial mechanism is not necessary to become established in the gut in the absence of an intact commensal microbial community.

E. coli is known to play important roles in resisting colonization of enteric pathogens in the phylum Proteobacteria. Our finding that the Tce1-mediated T6SS-3 pathway targets E. coli in vitro prompted us to further investigate whether it can facilitate Yptb overcoming colonization resistance in vivo through antagonism of resident gut E. coli. Therefore, mice were treated with streptomycin for 24 h to reduce the number of indigenous commensal bacteria, followed by oral inoculation of mice with 5 × 10^8 CFUs of E. coli DH5α. After 24 h, mice that had been colonized with E. coli were challenged with 5 × 10^9 CFUs of WT Yptb or Δtce1. At both 24 and 48 h after infection, the E. coli intestinal load of mice challenged with WT Yptb was significantly lower than that of mice challenged with Δtce1. By contrast, WT Yptb exhibited significantly higher levels of colonization in mice pre-colonized with E. coli commensals relative to Δtce1 (Fig. 5c, d and Supplementary Fig. 12d, e). These results demonstrated that...
the Tce1-mediated T6SS killing pathway plays a crucial role in overcoming colonization resistance through antagonism of commensal *E. coli*.

To gain further insight into the role of Tce1 in gut colonization, we investigated whether the antibacterial activity of Tce1 is directed against enteric pathogens such as *S. Typhimurium* that share the niche of *Yptb*. Streptomycin-treated mice were orally co-infected with 5 × 10⁸ CFUs of WT *Yptb* or Δtce1 or 5 × 10⁸ CFUs of *S. Typhimurium*. The *Yptb* and *S. Typhimurium* levels were measured in the intestine of co-infected mice at 8 h post-infection. Again, a dramatic decrease in *Salmonella* loads were observed in the cecum and small intestine of mice co-infected...
Fig. 5 The Tce1-mediated T6SS killing pathway facilitates Yptb colonization of the mouse gut. a, b Mice with and without streptomycin pre-treatment in a (Strep−: WT n = 11; Δtce1 n = 11; ΔclpV3* n = 13 and Strep+: WT n = 8; Δtce1 n = 8; ΔclpV3* n = 8) and b (Strep−: WT n = 10; Δtce1 n = 13; ΔclpV3* n = 10 and Strep+: WT n = 7; Δtce1 n = 8; ΔclpV3* n = 8) were orally gavaged with 10^9 CFUs of the indicated Yptb strains. Animals were sacrificed 24 h after bacterial challenge, and bacterial loads in cecum (a) and small intestine (b) were measured. c, d Streptomycin-treated mice (n = 9–14) were colonized with 5 × 10^8 CFUs of E. coli for 24 h and then challenged with 5 × 10^8 CFUs of WT Yptb, Δtce1 or buffer control (buffer control: n = 9; WT: n = 11; Δtce1: n = 14). Animals were sacrificed 24 h after the challenge, and surviving E. coli (c) and Yptb (d) in the cecum and small intestine were counted. PBS mixed with E. coli was used as the negative control. e, f Streptomycin-treated mice were gavaged with a 1:1 mixture of S. Typhimurium and WT Yptb or Δtce1 mutant (buffer control: n = 6; WT: n = 14; Δtce1: n = 14). Animals were sacrificed 8 h after the challenge, and CFU of S. Typhimurium (e) and Yptb (f) in the cecum and small intestine were counted. PBS mixed with S. Typhimurium was used as the negative control. Each data point represents result from one mouse; error bars represent mean ± SD of recovered CFUs. Statistical analysis of all experiments was carried out using the two-sided Mann–Whitney test. Differences were determined as significant when P < 0.05.
with WT Yptb but not with Δtce1. In contrast, the WT Yptb levels were higher than Δtce1, especially in the cecum (Fig. 5e, f), indicating that the Tce1-mediated T6SS killing pathway plays a crucial role in niche competition by targeting other enteric pathogens.

Discussion

Here, we report a non-canonical T6SS killing pathway in Yptb in which a small nucleolar effector, Tce1, can be translocated into the recipient cell in a contact-independent manner. This is in sharp contrast to the contact-dependent delivery by the T6SSs that directly translocate effectors across the envelope of recipient cells35,26,36. Unlike canonical T6SS effectors, Tce1 can enter prey cells after being secreted to the extracellular medium (Figs. 2 and 4). Interestingly, T6SS-3 can also deliver Tce1 into target cells in a contact-dependent, receptor-independent manner likely with greater efficiency (Supplementary Fig. 11). This dual mode of translocation makes T6SS-3 an advanced bacterial weapon that functions not only on solid surfaces and in biofilms, but also in liquid culture, and might protect the attacking cells from harm due to retary T6SS attacks10 from target cells.

Although we have shown that BtuB and OmpF are important for Tce1 cell entry, the underlying translocation mechanisms remain unclear. Further work is needed to test whether Tce1 cell entry is mediated by mechanisms similar to those used by some bacteriocins, which use BtuB, OmpF or other OM proteins as receptors28–31,37. As numerous T6SS-related toxins have been detected in growth media previously2,4,38,39, it is tempting to speculate that other T6SS toxins may be able to enter target cells in a contact-independent manner.

Methods

Ethics statement. All mouse experimental procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China. The protocol was approved by the Animal Welfare and Research Ethics Committee of Northwest A&F University (protocol number: NWAUSFM20180001). Six-week-old female mice (BALB/c) were purchased from the central animal laboratory of Xi'an JiaoTong University (Xi'an, China) and kept in a temperature (24 ± 2 °C), 50 ± 10% humidity, airflow of 35 exchanges and light-controlled room (12 h light, 12 h darkness) with free access to food and water.

Bacteria strains and growth conditions. Bacteria strains and plasmids used in this study are listed in Supplementary Table 1. Yptb strains were grown in YLB (1% tryptone, 0.5% yeast extract, 0.5% NaCl) or M9 medium (Na2HPO4, 6 g L⁻¹; KH2PO4, 3 g L⁻¹; NaCl, 0.5 g L⁻¹; NH4Cl, 1 g L⁻¹; MgSO4, 1 mM; CaCl2, 0.1 mM; glucose, 26 g L⁻¹; or 30 °C. E. coli and S. typhimurium were cultured in LB broth at 37 °C or 26 °C. Appropriate antibiotics were included in growth medium and their corresponding concentrations were: Ampicillin (100 µg mL⁻¹), Nalidixic acid (20 µg mL⁻¹), Kanamycin (50 µg mL⁻¹), Tetracycline (5 µg mL⁻¹ for Yptb and 15 µg mL⁻¹ for E. coli), Gentamicin (20 µg mL⁻¹), Chloramphenicol (20 µg mL⁻¹).

Plasmid construction. Primers used in this study are listed in Supplementary Table 2. For obtaining expression plasmids, the genes encoding Yptb Tce1 (YPK_0954) was amplified by PCR. The DNA fragment was digested and cloned into similarly digested pGEX6p-1 and pET28a vectors, yielding corresponding plasmids derivatives. The expression clones of Tce1 (YPK_0955), BtuB (YPK_0782), OmpF (YPK_2801), Tci1 (YPK_0954) was amplified and inserted into pTargetF1, E. coli DH5α was transformed with pTargetF1–tce1 which was produced by PCR using primer pair B3966-up-F/B3966-up-R and B3966-down-F/B3966-down-R respectively. The DNA fragment that code for Tce1, BtuB, and OmpF was amplified and inserted into similarly digested pME6032 to generate pME6032-tce1-vsvg, pME6032-yptb-vsvg and pME6032-tci1 were constructed in the same way. The integrity of the insert in all constructs was confirmed by DNA sequencing.

In-frame deletion and complementation. To construct in-frame deletion mutants, pDM4 derivatives were transformed into Yptb through E. coli S17-1 mediated conjugational mating, achieved by mixing 50 µL volumes of overnight LB cultures of E. coli S17-1 λpir donor with the Yptb recipient. The mixture was spotted onto selective LB plate and incubated for 16 h at 30 °C. The DNA fragment of the introduced plasmid into Yptb chromosome by single cross-over was selected on YLB plates containing 20 µg mL⁻¹ chloramphenicol and 20 µg mL⁻¹ naldixic acid. The chloramphenicol-resistant colonies were grown overnight in LB broth allowing for a second cross-over to occur. Selection for loss of the genome integrated sucb-containing plasmid was performed on YLB plates containing 20% sucrose and 20 µg mL⁻¹ naldixic acid. Strains growing on this plate were tested for chloramphenicol sensitivity by parallel spotting on YLB plates containing either chloramphenicol or nalidixic acid. Chloramphenicol-resistant and sucrose-resistant colonies were tested for deletion by PCR and confirmed by DNA sequencing. For overexpression or complementation of Tce1, BtuB and OmpF strains, the pME6032 or pET28a derivatives were transformed into relevant Yptb strains by electroporation and the expression in Yptb was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG).

Clustered regularly interspaced short palindromic repeats with Cas9 (CRISPR-Cas9) system was used to construct deletion mutants in E. coli DH5α as described previously41, with the spectromycin resistance gene in the pTargetF plasmid replaced by a chloramphenicol resistance gene (pTargetF1). The Tce1 and Tce1S8A/A16E proteins, further processes were adopted after His-NTA resin purification. The eluted samples from Ni-NTA were desalted into QA PAGE. The plasmids pTargetF and pCas in the transformation were used to construct E. coli plasmid derivatives. The expression clones of Tci1 (YPK_0955), BtuB (YPK_0782), OmpF (YPK_2801), Tci1 (YPK_0954) was amplified and inserted into pTargetF1 using primer pair B3966-g20-F/B3966-g20-R. The DNA fragment of Tci1, BtuB, and OmpF was amplified and inserted into similarly digested pME6032 or pKT100 derivatives were transformed into relevant Yptb strains by electroporation and the expression in Yptb was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were collected and disrupted by sonication for another 12 h at 22 °C in a rotary shaker with a speed setting of 150 rpm. The DNA fragment of the introduced plasmid into Yptb chromosome by single cross-over was selected on YLB plates containing 20 µg mL⁻¹ chloramphenicol and 20 µg mL⁻¹ naldixic acid. The chloramphenicol-resistant colonies were grown overnight in LB broth allowing for a second cross-over to occur. Selection for loss of the genome integrated sucb-containing plasmid was performed on YLB plates containing 20% sucrose and 20 µg mL⁻¹ naldixic acid. Strains growing on this plate were tested for chloramphenicol sensitivity by parallel spotting on YLB plates containing either chloramphenicol or nalidixic acid. Chloramphenicol-resistant and sucrose-resistant colonies were tested for deletion by PCR and confirmed by DNA sequencing. For overexpression or complementation of Tce1, BtuB and OmpF strains, the pME6032 or pET28a derivatives were transformed into relevant Yptb strains by electroporation and the expression in Yptb was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG).

Overexpression and purification of recombinant proteins. To express and purify His- and GST-tagged recombinant proteins, pET28a and pGEX-26-1 derivatives were transformed into E. coli BL21(DE3) and E. coli XL-1 Blue. Bacteria were cultured in 5 ml LB at 37 °C to reach stationary phase and re-inoculated with a ratio of 1:100 to fresh LB, cultivated at 37 °C until OD₅₇₀ = 0.40. Then 0.2–0.3 mM IPTG was added into the growth medium, continue cultivation for another 12 h at 22 °C in a rotary shaker with a speed setting of 150 rpm. Cells were collected and disrupted by sonication and purified with the His-Bind Ni-NTA resin or GST-Bind Resin (Novagen, Madison, WI), respectively, according to manufacturer's instructions. Purified proteins were dialyzed against PBS (phosphate-buffered saline) at 4 °C overnight. To get highly purified His-tagged Tce1, BtuB and OmpF, the cleaved His-Bind Ni-NTA resins were purified with the eluted samples from Ni-NTA were desalted into QA buffer (20 mM Tris-HCl, 0.2 M NaCl, 10% glycerol, pH 7.5) and loaded onto Hitrap Q HP 1 mL using AKTA Pure 25 chromatography system (GE Healthcare, USA). A 20 ml salt concentration gradient from 0.2 to 1 M NaCl was performed further for purification. After His-Bind Ni-NTA purification, the eluted proteins were examined by SDS–PAGE. To express and purify outer membrane proteins, E. coli BL21(DE3) that contains the corresponding expression vector was grown in 5 ml LB at 37 °C and transferred to 250 ml LB until OD₅₇₀ reached 0.4. 0.3 mM IPTG was added and the growth condition of bacteria was shifted to 22 °C with shaking at 150 rpm. Incubated cells were collected and resuspended in binding buffer (20 mM Tris-HCI, 150 mM NaCl, 10% glycerol, pH 7.5) and loaded onto Hitrap Q HP 1 mL using AKTA Pure 25 chromatography system (GE Healthcare, USA). A 20 ml salt concentration gradient from 0.2 to 1 M NaCl was performed further for purification. After His-Bind Ni-NTA purification, the eluted proteins were examined by SDS–PAGE.
100 mM glycine, pH 8.3) with 8 M urea, and then it was centrifuged again to remove the residual membranes. The supernatant was purified with the Hi-Prep Ni-NTA resin and eluted with elution buffer. The denatured protein was mixed with refolding buffer (55 mM Tris-HCl, 0.21 mM NaCl, 0.88 mM KCl, 880 mM L-arginine, 0.5% SB-12, pH 7.0) with the ratio of 1/20 followed by 4 °C overnight incubation. The refolded protein was ultrafiltered to increase its purity and concentration and then dialyzed with buffer containing 55 mM Tris-HCl (pH 6.5), 0.2 mM EDTA and 0.05% BSA for 4 h at 4 °C, and then 50 mM other divalent metal or other component was added in the reaction system as indicated in different experiments. The reaction of DNA hydrolysis was carried out at 37 °C for 30 min or indicated time points and the integrity of DNA was analyzed by 0.7% agarose gel electrophoresis.

**Dhaft assay.** Total RNA was extracted from E. coli T1G and tRNA from E. coli BL21(DE3) (Roche, Germany, catalog no. 10190541001) was purchased from Sigma-Aldrich. Two micrograms of RNA was incubated with different concentrations of Tcet in same reaction system as DNase assay at 37 °C for 30 min. The integrity of RNA was detected by 2% agarose gel.

**Fluorophore labeling of proteins.** Fluorophore labeling of proteins was performed as described with minor modifications. Cysteine residues were present in the C-terminus of both Tcet and Tscet. To prepare the proteins for subsequent labeling reactions, 5 mM DTT was used to reduce the potential disulfide bonds formed by these cysteine residues, and the reactions were conducted at room temperature for 2 h. After the reduction of disulfide bonds, DTT is removed by dialysis in 20 mM potassium phosphate (pH 7.0) and 500 mM NaCl. Labeling reactions were carried out by adding 10 mM maleimide fluorophores (Thermo Fisher Scientific, USA) and the Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, USA) was used to measure mRNA abundance in each of the samples according to manufacturer’s instructions. Primers used in this study are list in Supplementary Table 2.

**Fluorescent labeling of live bacteria.** Fluorescent labeling of live bacterial strains was performed according to described methods with some modifications. Briefly, cultures at OD600 = 0.7 were centrifuged and resuspended in M9-glucose containing 1 μM fluorophore-conjugated protein, incubated in the dark at room temperature for 30 min. The cells were washed five times to remove the free label and resuspended in 100 μl volume in M9-glucose. Ten microcells of the filter suspensions was stained with 1% (w/v) agarose pads on a microscope slide before sealing with a clean glass coverslip. The result was obtained by high-speed rotary disc type fluorescence confocal microscope (Andor Revolution-XD, UK).

**TUNEL (terminal deoxynucleotidyl) transference dUTP nick-end labeling and flow cytometry analysis.** Overnight culture of E. coli BL21(DE3) containing the pET28a plasmid and its derivatives expressing Tcet alone (pET28a-tcet) or Tscet-Tcet together (pET28a-tcet-tcet) was diluted 100-fold into LB broth and incubated at 26 °C with 180 rpm shaking. After incubated at 26 °C for 2 h, the expression of toxin and immunity genes was induced by addition of 0.5 mM IPTG and continue cultivation for 4 h at 26 °C. Collected cells were washed with PBS, fixed, incubated for 5 min in PBS with 0.3% Triton X-100 and stained using One-step Staphylococcus aureus apoptosis detection kit (Beiyi). When genomic DNA breaks, exposed 3'-OH can be labeled with green fluorescent probe FITC catalyzed by terminal deoxynucleotidyl transferase (TdT), which can be detected by flow cytometry (Beckman, CytoFLEX). Ten-thousand cells were gathered for each sample and analyzed by FlowJo, version 10.

**DAPI staining and flow cytometry analysis.** To perform DAPI staining and flow cytometry analysis, overnight culture of E. coli BL21(DE3) containing the pET28a plasmid or its derivatives expressing Tcet alone (pET28a-tcet) or Tcet-Tcet together (pET28a-tcet-tcet) were diluted 100-fold into LB broth and incubated at 37 °C with 150 rpm shaking. After incubated at 37 °C for 4 h, the membrane was washed five times in TBST buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.4), then incubated with 1:5,000 diluted horseradish peroxidase conjugated secondary antibodies (Shanghai Genomics, catalog no. DY60030, lot number: 200614) for 4 h at 4 °C, and washed five times with TBST buffer. Signals were detected by using the ECL plus kit (GE Healthcare, Piscataway, NJ) with a Chemiluminescence imager (Tanon 5200Multi, Beijing).
26 °C with 180 rpm shaking. After incubated at 26 °C for 2 h, the expression of toxin and immunity genes was induced by addition of 0.5 mM IPTG and continue cultivation for 4 h at 26 °C. Collected cell was washed with PBS, fixed, incubated for 5 min in PBS with 0.3% Triton X-100 stained using 10 μg ml⁻¹ DAPI for 30 min at 37 °C (Solarbio, China), then washed three times with PBS and detected by fluorescence microscope (Andor Revolution-XD, Britain) or flow cytometry (Beckman, CytoFLEX). Twenty-thousand cells were gathered for each sample and analyzed by FlowJo V10.

**Subcellular fractionation.** To perform subcellular fractionation [50], 2 ml overnight grown Yptb culture (OD₆₀₀ 1.0) was collected, washed, and incubated in 2 ml M9 containing 0.05 mg Tce1 at 30 °C for 60 min. Tce1-treated bacterial cells were washed with PBS to remove extracellular Tce1 protein, and incubated into 285 μl sucrose buffer (20 mM PBS, pH 7.4, 20% sucrose, 2.5 mM EDTA) for 20 min at room temperature. After that, 285 μl ice-cold 0.5 mM MgCl₂ was added and incubated for 5 min with gentle agitation. The suspension was centrifuged at 7000 × g for 20 min at 4 °C to collect the supernant containing periplasmic proteins (Peri). The pellet was resuspended in SDS-loading buffer and defined as cytoplasmic (Cyto). All the samples were examined by SDS–PAGE and western blotting analysis.

**Construction of mutant library by epPCR.** Error-prone PCR (epPCR) was conducted on plasmid PET28a-tcel by using the QuickMutation™ Random Mutagenesis Kit (Beyotime Biotechnology, China) with primers tcel-F- BamHI and tcel-R-SflI according to manufacturer's instructions. The epPCR program was as follows: 94 °C for 3 min, 30 cycles of 30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C, followed by 30 min at 72 °C final extension. The PCR products were gel-purified, digested with BamHI and SflI, and cloned into similarly digested PET28a. The ligation mixture was transformed into BL21(DE3). Transformants lost toxicity were screened in LB medium containing 0.5 mM IPTG and were further verified by cloning the mutated alleles of tcel into new vector[51]. The mutations were identified by DNA sequencing analysis.

**Intra-species and inter-species competition in vitro.** For intra-species competition assays[52], overnight grown strains were washed and adjusted to OD₆₀₀ of 1.0 with M9 medium before mixing for competition. The initial donor-to-recipient ratio was 1:1 and the co-cultures were either spotted onto a 0.22 μm nitrocellulose membrane (Nalgene) placed on M9 agar plates at 26 °C for 48 h (for contact-dependent competition), or inoculated into 2 ml M9 medium at 26 °C with shaking for 24 h or 48 h (for contact-independent competition in liquid medium). For contact-independent competition performed on a solid surface, 5 μl of the recipient strain was spotted on 0.22 μm nitrocellulose membrane on M9 agar plates. After the bacterial solution was dried, another 0.22 μm nitrocellulose membrane was put on it and 5 μl of the donor strain was spotted on the same place of the second membrane and incubated at 26 °C for 48 h. The donor and recipient strains were labeled by pKT100 (KmR) and monitored for 24 h or 48 h (for contact-independent competition in liquid medium). The initial donor-to-recipient ratio was 1:1 and the co-cultures were either spotted onto a 0.22 μm nitrocellulose membrane (Nalgene) placed on M9 agar plates. After the bacterial solution was dried, another 0.22 μm nitrocellulose membrane was put on it and 5 μl of the donor strain was spotted on the same place of the second membrane and incubated at 26 °C for 48 h. The donor and recipient strains were labeled by pKT100 (KmR) or pACYCl84 (CmR), respectively, to facilitate screening on YLB plates. At indicated time points after the competition, the CFU ratio of the donor and recipient strains was measured by plate counts. Data from all competitions were analyzed using the Student's t-test. The mean of triplicate experiments was used for calculation.

**Murine infection and in vivo competition assays.** Female 6-week-old BALB/c mice were adapted in the lab for 3 days and orally gavaged with 10⁹ CFUs of the indicated Yptb strains harboring pKT100 (KmR) or E. coli DH5α or S. Typhimurium strains containing pBBRMCS5-GFP (GmR) or pME6032 (TetR) (gentamycin or tetracycline resistant) were washed three times with M9 medium, and adjusted to OD₆₀₀ 1.0. Yptb strains diluted to 10-folds and target strains attenuated to 100-folds were mixed together so that the ratio of donor and recipient was 10 to 1 in M9 liquid, incubated at 26 °C with the speed of 120 rpm. After the competition, mixtures were serially diluted, counted on LB plates containing appropriate antibiotics, and the final CFU was determined.

**Data availability**

The protein sequences are available from the Uniprot database (http://www.uniprot.org/). Other data supporting the findings of this study are included in the article and its Supplementary Information files, or from the corresponding authors upon request. Source data are provided with this paper.

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**Statistics and reproducibility.** Statistical analyses were performed using GraphPad Prism Software (GraphPad Prism 7.00). All experiments were performed at least three independent replicates. Statistical analyses of colonization assay in mice, intra-species and inter-species competition assay in mice were analyzed using two-sided Mann–Whitney test. All other experiments were analyzed using unpaired, two-tailed Student's t-test. Statistical significance is determined when *P* < 0.05. All gels, blots, and micrographs were repeated for at least three times with similar results.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.
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## Competing interests
The authors declare no competing interests.

## Additional information

### Supplementary information
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