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ZntR positively regulates T6SS4 expression in *Yersinia pseudotuberculosis*[§]

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The type VI secretion system (T6SS) is a widespread and versatile protein secretion system found in most Gram-negative bacteria. Studies of T6SS have mainly focused on its role in virulence toward host cells and inter-bacterial interactions, but studies have also shown that T6SS4 in *Yersinia pseudotuberculosis* participates in the acquisition of zinc ions to alleviate the accumulation of hydroxyl radicals induced by multiple stressors. Here, by comparing the gene expression patterns of wild-type and *zntR* mutant *Y. pseudotuberculosis* cells using RNA-seq analysis, T6SS4 and 17 other biological processes were found to be regulated by ZntR. T6SS4 was positively regulated by ZntR in *Y. pseudotuberculosis*, and further investigation demonstrated that ZntR regulates T6SS4 by directly binding to its promoter region. T6SS4 expression is regulated by zinc via ZntR, which maintains intracellular zinc homeostasis and controls the concentration of reactive oxygen species to prevent bacterial death under oxidative stress. This study provides new insights into the regulation of T6SS4 by a zinc-dependent transcriptional regulator, and it provides a foundation for further investigation of the mechanism of zinc transport by T6SS.

Keywords: *Yersinia pseudotuberculosis*, Type VI secretion system, ZntR, oxidative stress, ROS homeostasis

Introduction

Bacteria have evolved several specialized secretion systems to transport proteins or DNA across membranes to the extracellular milieu or host cells in response to specific environmental cues. In Gram-negative bacteria, six secretion systems have been described or defined in terms of their structure and transport mechanism (Holland, 2010). In 2006, the type VI secretion system (T6SS) was characterized in *Vibrio cholerae* using the *Dictyostelium* host model system (Pukatzki *et al.*, 2006). Structurally, T6SS has 13 core components; additional components may also be present (Cascales and Cambillau, 2012). ClpV, a member of the AAA^+ protein family (ATPases associated with various cellular activities) with an ATP-binding domain that hydrolyzes ATP to provide energy, is essential for secretion (Bingle *et al.*, 2008). Hemolysin-regulated protein (Hcp) and valine-glycine repeat protein G (VgrG) form a needle- or spike-like structure to transport proteins (Mougous *et al.*, 2006). Electron microscopy revealed that TssBC can assemble into cogwheel-like structures on Hcp, with an observable tubular configuration resembling the bacteriophage sheath (Basler *et al.*, 2012). In some systems, a protein containing a forkhead-associated domain post-translationally regulates T6SS via a threonine phosphorylation signaling cascade (Mougous *et al.*, 2007).

T6SS is involved in various functions, but most previous studies have focused on virulence toward host cells and the export of anti-bacterial proteins (Russell *et al.*, 2014). In *V. cholerae*, VgrG1 harbors an actin crosslinking domain that when transported into the cytosol of macrophages by T6SS abolishes actin crosslinking and improves bacterial survival (Ma *et al.*, 2009). *Agrobacterium tumefaciens* uses T6SS to translocate Tde, an antibacterial DNase, to attack neighboring bacteria within plant hosts (Ma *et al.*, 2014). *Pseudomonas aeruginosa* uses T6SS to deliver two effector proteins, Tse1 and Tse3, to the periplasm of recipient cells; they hydrolyze peptidoglycan to provide a competitive fitness advantage to *P. aeruginosa*. Tsi1 and Tsi3 are periplasmically-localized immunity proteins that protect *P. aeruginosa* from being lysed by Tse1 and Tse3 (Russell *et al.*, 2011). Weber *et al.* (2009) found that T6SS in *Vibrio anguillarum* is positively regulated by the stress-response regulator RpoS and quorum sensing regulator VanT. In addition, it plays a role in the signal sensing mechanism of the general stress response (Weber *et al.*, 2009). In *Yersinia pseudotuberculosis*, T6SS4 is essential for survival under acidic conditions; expression is regulated by the EnvZ-OmpR two-component regulatory system (Gueguen *et al.*, 2013; Zhang *et al.*, 2013). The activation of T6SS has been shown to depend on the osmolarity of the milieu in *V. cholerae* O1 (Ishikawa *et al.*, 2012). Under acidic conditions, T6SS is activated by an ExoR-ChvG/ChvI cascade in *A. tumefaciens* (Wu *et al.*, 2012). Previously, we discovered that in *Y. pseudotuberculosis* T6SS4 imports zinc ions (Zn^{2+}) into bacterial cells from the environment, which alleviates the buildup of hydroxyl radicals induced by multiple stressors (Wang *et al.*, 2015).

Zinc is a trace element found in proteins and enzymes that is required for the growth and survival of bacteria (Bobrov *et al.*, 2014). Intracellular ion homeostasis mitigates the risk

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from reactive oxygen species (ROS), which are induced by multiple stressors during the bacterial life cycle (Faulkner and Helmann, 2011; Mols and Abee, 2011). Zinc is an important factor in ROS homeostasis as it competes with iron in the Fenton reaction (Faulkner and Helmann, 2011). As excessive zinc can be toxic to cells, its concentration must be precisely regulated by different regulators. Zinc uptake regulator (Zur) is a ferric uptake regulator (Fur) family transcriptional regulator that functions as a repressor of the zinc uptake system of ZnuABC and ZinT (Patzer and Hantke, 1998; Petrarca *et al.*, 2010). Zur binds the Zur box in the promoter to inhibit gene expression, thereby preventing zinc uptake under high zinc conditions (Panina *et al.*, 2003). ZntR is a member of the MerR family of metal-responsive transcriptional regulators of ZntA and ZitB in *Escherichia coli* (Pruteanu *et al.*, 2007; Wang *et al.*, 2012). ZntA, a P-type ATPase, is a primary zinc export system, and the transcription of *zntA* is activated by ZntR. The binding of zinc to ZntR converts it into a strong transcriptional activator, resulting in binding to the promoter of *zntA* and the increased efflux of zinc (Rensing *et al.*, 1997; Outten *et al.*, 1999). Transcription of the zinc efflux or uptake system is activated by numerous transcriptional regulators (Outten and O'Halloran, 2001; Wang *et al.*, 2012).

Yersinia pseudotuberculosis contains four T6SS gene clusters, which may play different roles in the life cycle of the bacterium (Bingle *et al.*, 2008). Previously, T6SS4 was found to have a direct role in Zn^{2+} acquisition by bacterial cells in order to mitigate the effect of hydroxyl radicals induced by multiple stressors, and it is regulated by OxyR, a global oxidative stress regulator (Wang *et al.*, 2015). In this study, we found that T6SS4 is also positively regulated by ZntR in *Y. pseudotuberculosis* based on RNA-seq analysis, and further investigation demonstrated that ZntR regulates T6SS4 by directly binding to its promoter region. T6SS4 is regulated by zinc through ZntR to maintain zinc homeostasis, and it controls the ROS level to prevent cell death under conditions of oxidative stress.

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Supplementary data Table S1. *E. coli* strains were grown at 37°C in LB with appropriate antibiotics. *Y. pseudotuberculosis* strains were cultured in Yersinia-Luria-Bertani (YLB) broth (tryptone 1%, yeast extract 0.5%, NaCl 0.5%) with appropriate antibiotics at 26°C. Bacterial growth was monitored by spectrophotometer. Construction and screening of mutants were done as described previously (Zhang *et al.*, 2013). For selection of mutant and complementary strains, in accordance with the vector used, antibiotics were added at the following concentrations: chloramphenicol, 20 μg/ml; kanamycin, 50 μg/ml; nalidixic acid, 20 μg/ml.

Plasmid construction

Primers used in this study are listed in Supplementary data Table S1. To construct the *zntR* mutant, the plasmid pDM4-Δ*zntR* was used. About 1,000 bp upstream and downstream fragments flanking *zntR* ORF were amplified with primer pairs *zntR*-1F-*Bgl*II/*zntR*-1R and *zntR*-2F/*zntR*-2R-*Sal*I, respectively, and the amplified fragments were ligated by overlapping PCR. The PCR product was digested with *Bgl*II and *Sal*I and inserted into the corresponding site of suicide vector pDM4 to generate plasmid pDM4-Δ*zntR*.

To construct the complementary vector pKT100-*zntR*, primers *zntR*-F-*Sph*I, and *zntR*-R-*Sal*I were used to amplify *zntR* from *Y. pseudotuberculosis* genomic DNA. The PCR product was digested (*Sph*I/*Sal*I) and inserted into similarly digested pKT100 to construct plasmid pKT100-*zntR*, which is then electroporated into the Δ*zntR* mutant to generate the complementation strain. To express His_6-tagged ZntR, primers *zntR*-F-*Bam*HI and *zntR-R-Sal*I were used to amplify *zntR* from template. The PCR product was digested (*Bam*HI/*Sal*I) and inserted into the similarly digested expression vector pET28a, resulting in a plasmid pET28a-*zntR*. All the plasmids constructed above were confirmed by endonuclease digestion and DNA sequencing.

RNA-seq experiments

The exponentially growing cells of the two strains were used for RNA-Seq analysis. All experiments were performed in triplicate. For each biological sample, total RNA samples were extracted using the Bacterial mRNA Enrichment kit (Life Technologies) and then was examined with an Agilent 2100 Bioanalyzer, at least 20 μg of RNA were used for cDNA library preparation. Illumina sequencing was performed at the Beijing Genomic Institute (BGI-Shenzhen). Briefly, after removing residual DNA and rRNA, cDNA was synthesized using random hexamer primer and reverse transcriptase based on the mRNA-enriched RNA. After the end preparation and ligation of adaptors, the products were amplified by PCR and purified to create the final cDNA library. Libraries were sequenced on an Illumina HiSeqTM 2000 platform. The undesired raw reads including the reads with adaptors, reads with unknown base more than 5%, and low-quality reads, were removed to obtain clean reads. Then, the clean reads of the strains were mapped to reference genome of *Y. pseudotuberculosis* (http://www.genome.jp/kegg-bin/show_organism?org=ypy) using BWA alignment (Li and Durbin, 2009). RPKM (number of reads per kilobase of exon region per million mapped reads) was used to normalize the expression level of genes by RESM software (Mortazavi *et al.*, 2008; Li and Dewey, 2011). The tool NOISeq was used to identify the DEGs between wild type and the Δ*zntR* mutant (Tarazona *et al.*, 2011). For the DEGs, the KEGG pathway enrichment analysis of DEGs was also performed to identify significantly enriched metabolic pathways using the KEGG Orthology (KO) system (Kanehisa *et al.*, 2008).

Quantitative real-time PCR

Bacterial cultures were harvested during the mid-exponential phase and RNA was extracted using the EasyPure RNA Kit (TransGen Biotech) and treated with RNase-free DNase (TransGen Biotech). The purity and concentration of the RNA were determined by gel electrophoresis and spectrophotometry (Epoch, BioTek Instruments). First-strand cDNA

was reverse transcribed from 500 ng total RNA with the TransScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech). Quantitative real-time PCR (qRT-PCR) was performed in CFX96 Real-Time PCR detection system (Bio-Rad) with TransStart Green qPCR SuperMix (TransGen Biotech). For all primer sets (Supplementary data Table S1), the following cycling parameters were used: 95°C for 30 sec followed by 40 cycles of 94°C for 15 sec, 50°C for 30 sec. Assay was performed in triplicate and for standardization of results, the relative abundance of 16S rRNA was used as the internal standard.

Construction of chromosomal fusion reporter strains and β-galactosidase activity assays

The *lacZ* fusion reporter vector pDM4-T6SS4*p*::*lacZ* was transformed into *E. coli* S17-1 and then transferred to wild-type and Δ*zntR* mutant strains by conjugation as described previously (Zhang *et al.*, 2013). To construct the *lacZ* fusion reporter vector pDM4-*zntAp*::*lacZ*, primers *zntAp*-F-*Sal*I/*zntAp*-R-*Xba*I were used to amplify the 557 bp *zntA* promoter fragment from *Y. pseudotuberculosis* genomic DNA. The PCR product was digested with *Sal*I/*Xba*I and inserted into similarly digested pDM4-*T6SS4p*::*lacZ* to produce pDM4-*zntAp*::*lacZ*. To construct T6SS4 promoter with mutations in the ZntR binding region, overlap PCR was performed to replace the region with identical amount of irrelevant base pairs. Briefly, to replace the ZntR binding site, primer pairs T6SS4*p*-F-*Sal*I/T6SS4*p*M_{ZntR}-R and T6SS4*p*M_{ZntR}-F/T6SS4*p*-R-*Xba*I were used to amplify the up-fragment and down-fragment of T6SS4 promoter, respectively. Overlap PCR was carried out by using the up-fragment and down-fragment as templates and T6SS4*p*-F-*Sal*I/T6SS4*p*-R-*Xba*I as primer pair to obtain the DNA fragment T6SS4*p*M_{ZntR}. This fragment was further digested with *Sal*I and *Xba*I and inserted into similarly digested pDM4-T6SS4*p*::*lacZ* to construct pDM4-T6SS4*p*M_{ZntR}::*lacZ*. The *lacZ* fusion reporter strains were grown in the corresponding conditions and *o*-nitrophenyl-β-galactoside (ONPG) was used as the substrate for β-galactosidase activity assay (Miller, 1992).

Protein expression and purification

In order to express and purify His_6-tagged recombinant ZntR protein, the expression vector pET28a-*zntR* was transformed into *E. coli* BL21 competent cells. Overnight-grown culture was inoculated into the fresh LB broth and cultured at 37°C to OD_{600}=0.5. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 0.4 mM, and further incubated at 26°C for 10 h. Cells were harvested, disrupted by sonication and then purified with the His·Bind Ni-NTA resin (Novagen) according to the manufacturer's instructions. Purified recombinant proteins were dialyzed against the appropriate buffer at 4°C for 4 h and stored at -80°C until used. The purity of the purified protein was verified as >90% homogeneity based on SDS-PAGE analysis and protein concentrations were determined by the Bradford assay.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described previously (Wang *et al.*, 2015). The biotin-labeled DNA probes were amplified from the *T6SS4* promoter regions, with the biotin-labelled primers T6SS4*p*-ZntR-F-5′Biotin/T6SS4*p*-ZntR-R-5′Biotin, using plasmids pDM4-T6SS4*p*::*lacZ* or pDM4-T6SS4*p*M_{zntR}::*lacZ*. For negative control, the unlabeled competitor DNA was amplified from the same template using the unlabelled primers T6SS4*p*-ZntR-F/T6SS4*p*-ZntR-R. Also, an unrelated DNA in the similar length and an unrelated protein BSA were included in the binding assay system to serve as negative controls. All PCR products were purified by Universal DNA Purification Kit (TIANGEN). Each 20-μl EMSA reaction solutions were prepared by adding the following components according to the manufacturer's protocol (LightShift Chemiluminescent EMSA kit, Thermo Fisher Scientific): 1×binding buffer, 50 ng poly(dI-dC), 2.5% glycerol, 0.05% NP-40, 5 mM $MgCl_2$, 20 fmol Biotin-DNA, 4 pmol unlabeled DNA as competitor and different concentrations of proteins. Reaction solutions were incubated for 20 min at room temperature. The protein-probes mixture was separated with a 5% polyacrylamide native gel and transferred to an immobilon-Ny+ membrane (Millipore). The biotin-labeled probes were detected by streptavidin-horseradish peroxidase conjugates and chemiluminescent substrate according to the manufacturer's protocol.

Stress survival assay

Y. pseudotuberculosis and its variant strains grown in YLB medium to mid-exponential phase were collected, diluted 50-fold into M9 medium containing 1.5 mM H_2O_2 and then incubated at 26°C for 1 h. After treatment, the cultures were serially diluted and plated on YLB agar plates. Colonies were counted after 20 h growth at 26°C. Percentage survival was calculated by dividing number of CFU of stressed cells by number of CFU of cells without stress. The assays were performed in triplicate.

Fluorescence dye-based intracellular ROS detection

To detect intracellular ROS, the fluorescent reporter dye 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetylester (CM-H_2DCFDA, Invitrogen) was used as previously described (Dong *et al.*, 2015; Wang *et al.*, 2015). Briefly, 1 ml samples were collected after treatment and then resuspended in 1 ml of M9 medium containing 10 μM CM-H_2DCFDA. Samples were incubated in dark for 20 min. The cells were then pelleted, the supernatant removed, and re-suspended in 1 ml filter-sterilized PBS. The resultant cell suspension (200 μl) was transferred to a dark 96-well plate. Fluorescence signals were measured using a SpectraMax M2 Plate Reader (Molecular Devices) with excitation/emission wavelengths of 495/520 nm. The results represent the mean of the representative assay performed in triplicate and the error bars represent the standard deviation.

Statistical analysis

Statistical analyses of gene expression data, LacZ activity, survival assay, ROS determination and expression data were performed using paired two-tailed Student's *t*-test. Statistical analyses were performed using GraphPad Prism software (GraphPad Software).

Results

Genome-wide analysis of the genes regulated by ZntR

ZntR, a MerR-family transcriptional regulator, is a zinc-sensitive regulatory protein found in a variety of bacteria; it affects the expression of several genes associated with zinc homeostasis. To identify the ZntR-dependent genes expressed in *Y. pseudotuberculosis*, RNA was extracted for RNA-seq from wild-type and Δ*zntR* mutant strains grown to the mid-exponential phase. After the quality control and based on the gene expression levels calculated by FPKM (Fragments Per Kilobase of transcript per Million mapped reads), 103 gene candidates with the differentiated expressions were found between wild-type and *zntR* mutant, including 46 up-regulated genes and 57 down-regulated genes (Supplementary data Table S2). Next, qRT-PCR data for ten representative genes were used to validate the RNA-seq data; the log_2-transformed values for all genes were in good agreement with the log_2-transformed fold changes observed in the RNA-seq data (Fig. 1A), signifying that the RNA-seq data are credible. Finally, the functions of the differentially expressed genes were identified using KEGG pathway analysis. As shown in Fig. 1B, 18 pathways were found in the differentially expressed genes, including those related to metabolism, environmental information processing, cell motility, secretion systems, and ABC transporters.

Differentially expressed genes related to T6SS

T6SS is a newly described secretion system found in many bacteria (Bingle *et al.*, 2008; Jani and Cotter, 2010). Our RNA-seq data revealed that the genes in the T6SS4 operon were down-regulated to 51.91% to 68.70% (Table 1). Hcp, which forms the hexameric rings for the T6SS needle structure, was down-regulated to 62.24%. VgrG was down-regulated to 68.70%. IcmF, a periplasmic domain protein, was down-regulated to 66.92%. TssB and TssC, which serve as tail sheath proteins in T6SS, were down-regulated to 60.08 and 51.91%, respectively. Similar results were observed for other structural genes. These data suggest that T6SS4 is positively regulated by the transcriptional regulator ZntR.

ZntR positively regulates the expression of T6SS4

To verify the role of ZntR in the expression of T6SS4, a single-copy T6SS4*p*::*lacZ* fusion was introduced into the chromosomes of wild-type, Δ*zntR* mutant, and complemented strain Δ*zntR*(*zntR*). The LacZ activity of the resulting strains was quantitatively measured (Fig. 2A). Compared to the wild-type strain, the T6SS4*p*::*lacZ* fusion decreased significantly in Δ*zntR*, and this decrease could be reversed by the complementary plasmid pKT100-*zntR*, confirming that ZntR positively regulates T6SS4 expression. Positive regulation of T6SS4 by ZntR was further confirmed using qRT-PCR analysis (Fig. 2B), which indicated that the expression of T6SS4

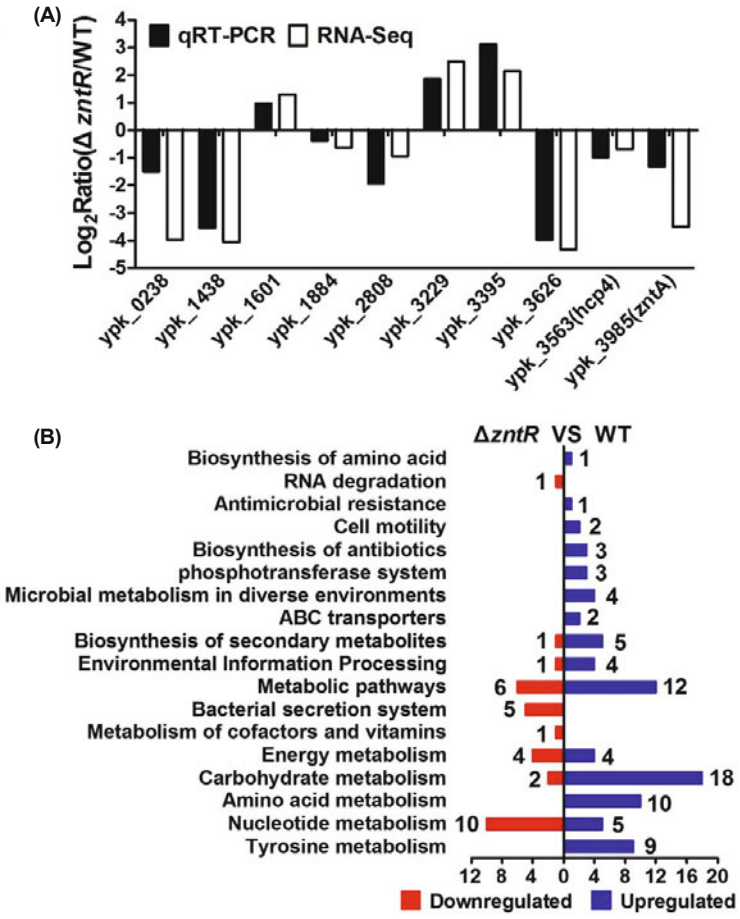

Fig. 1. Microarray analysis of ZntR regulated genes in *Yersinia pseudotuberculosis*. (A) Validation of RNA-Seq data using qRT-qPCR. Ten representative genes were evaluated for validation of the RNA-seq data using qRT-PCR. (B) KEGG pathway analysis of differentially expressed genes (*zntR* mutant vs wild-type). The red and blue bars represent down- and up-regulated genes, respectively, and the numeric labels represent the number of genes related to that pathway.

Table 1. Differentially expressed genes of T6SS4 in *Y. pseudotuberculosis*

Locus tag	Product	Gene description	Percentile[a] (100%)	Probability
ypk_3550	IcmF	Type VI secretion system protein ImpL	64.68	0.75
ypk_3551	ImpK	Type VI secretion system protein ImpK	59.77	0.83
ypk_3552	ImpL	Type VI secretion system protein ImpJ	67.36	0.75
ypk_3554		Conserved hypothetical protein	67.00	0.77
ypk_3555		Conserved hypothetical protein	60.11	0.83
ypk_3556		Pentapeptide repeat protein	58.49	0.80
ypk_3557		Pentapeptide repeat protein	65.69	0.75
ypk_3558	VgrG	Type VI secretion system secreted protein VgrG	68.70	0.76
ypk_3561	ImpG	Type VI secretion system protein ImpG	54.86	0.82
ypk_3562	ImpF	Type VI secretion system protein ImpF	66.92	0.75
ypk_3563	Hcp	Type VI secretion system secreted protein Hcp	62.24	0.85
ypk_3564	ImpC	Type VI secretion system protein ImpC	63.50	0.83
ypk_3565	TssB	Type VI secretion system protein TssB	60.08	0.86
ypk_3566	TssC	Type VI secretion system protein TssC	51.91	0.89

Percentile: (Δ*zntR*/WT)*100%

component genes *clpV4*, *hcp4*, and *yezP* was reduced in Δ*zntR*, and this decrease could be reversed by introducing the complementary plasmid pKT100-*zntR*. In contrast, the expression of T6SS1-3 in *Y. pseudotuberculosis* was not significantly affected in the Δ*zntR* mutant, indicating that ZntR regulates T6SS4 expression specifically (Fig. 2C).

ZntR binds directly to the T6SS4 promoter

To test whether regulation by the ZntR regulator was direct, an electrophoretic mobility shift assay (EMSA) was performed using the T6SS4 promoter region and purified His_6-ZntR. Incubation of labelled T6SS4 promoter probe with His_6-ZntR led to the formation of DNA-protein complexes (Fig. 3). Excessive unlabeled probe repressed the formation of the DNA-protein complex, while an unrelated DNA fragment did not (Fig. 3A). A putative ZntR binding motif was identified in the T6SS4 promoter by comparing the T6SS4 promoter with several reported ZntR-regulated promoters (Figs. 3B and S2) (Outten *et al.*, 1999; Brown *et al.*, 2003; Permina *et al.*, 2006). Replacement of this region with an irrelevant DNA sequence prevented the formation of DNA-protein complexes in the EMSA (Fig. 3A) and led to a significant reduction in promoter activity in wild type (Fig. 3C). Thus, the ZntR regulator specifically recognizes, binds to, and activates the T6SS4 promoter.

T6SS4 is responsive to zinc conditions in a ZntR-dependent manner

Escherichia coli ZntR acts as a hypersensitive transcriptional switch, regulating target genes in response to Zn^{2+} and other metal ions (Khan *et al.*, 2002). *Yersinia pseudotuberculosis* T6SS4 contributes to the environmental adaptability of the bacterium, and previous studies have indicated that T6SS4 is regulated by zinc and involved in zinc uptake (Gueguen *et al.*, 2013; Zhang *et al.*, 2013; Wang *et al.*, 2015). To test the hypothesis that T6SS4 may be regulated by zinc conditions via ZntR, the chromosomal reporter fusion strains WT (T6SS4*p::lacZ*) and Δ*zntR*(T6SS4*p::lacZ*) were grown under different zinc conditions using media with various concentrations of the zinc chelator N,N,N′,N′-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) or excess Zn^{2+}. As shown in Fig. 4A, in the wild-type strain, T6SS4 promoter activity was significantly up-regulated under zinc-depleted conditions triggered by TPEN, but down-regulated at high Zn^{2+} concentrations. Similar changes were found in the *zntR* mutant strain, but these were less significant than in the wild-type strain (Fig. 4B). Significant expression of *zntA* was induced by excessive zinc in the wild-type strain, but *zntA* expression did not change significantly in the *zntR* mutant (Fig. 4C). Expression of the *zntA* promoter exhibited similar patterns in the wild-type and mutant strains, but the

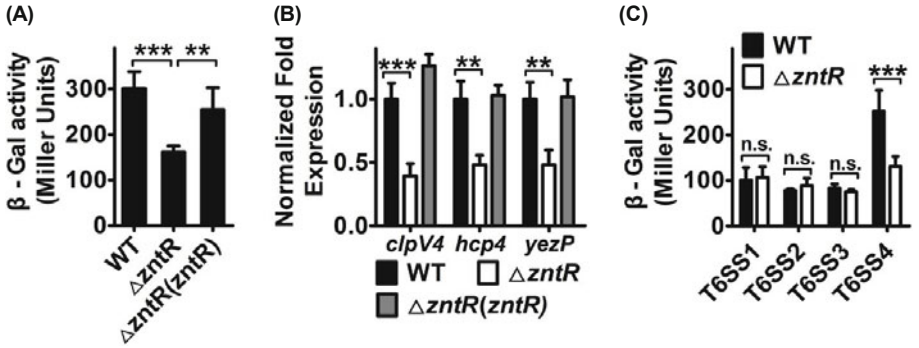

Fig. 2. ZntR positively regulates T6SS4 expression. (A) β-Galactosidase activity of the T6SS4 promoter in *Y. pseudotuberculosis* wild-type, Δ*zntR* mutant, and its complementary strain Δ*zntR*(*zntR*). (B) quantitative RT-PCR of indicated bacterial strains. The expression of T6SS4 components *clpV4*, *hcp4*, and *yezP4* were reduced in *zntR* mutant that could be restored by complementation. (C) β-Galactosidase analyses of T6SS1-4 promoter activities by using the transcriptional T6SS1-4*p::lacZ* chromosomal fusion reporters expressed in the wild-type and Δ*zntR* mutant. Data shown are the average of three independent experiments. The error bars indicate standard deviations of the mean. ***, $P<0.001$; **, $P<0.01$; n.s., not significant.

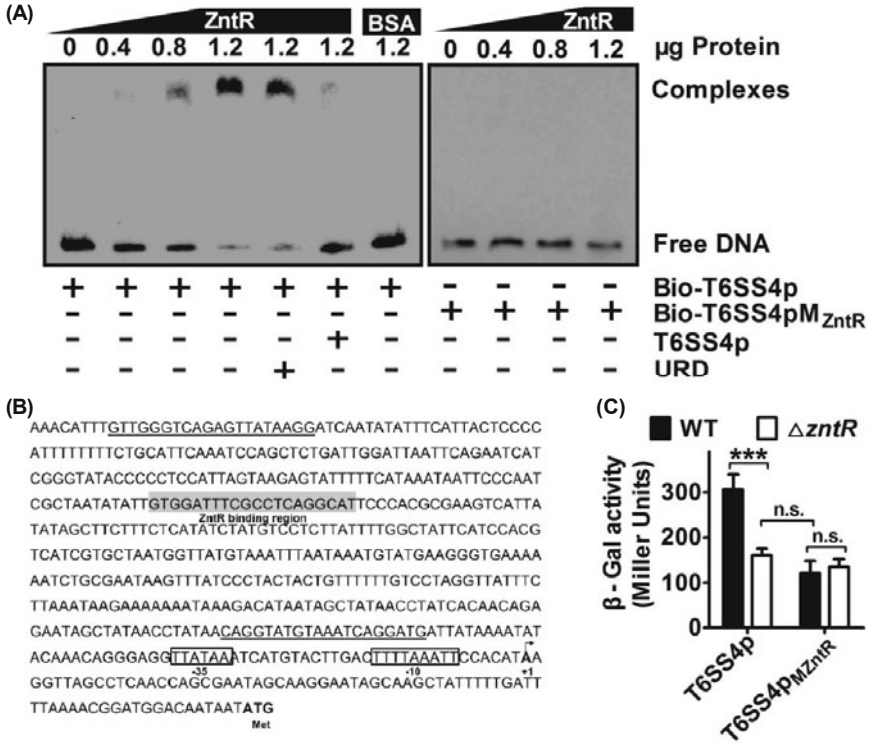

Fig. 3. ZntR directly binds to the T6SS4 promoter. (A) Biotin-labelled and/or unlabelled probe of T6SS4 or T6SS4pM$_{zntR}$ promoter was incubated with ZntR protein. The protein-DNA complexes were detected by streptavidin conjugated HRP and chemiluminescent substrate. Unlabelled and unrelated promoter was added to determine the binding specificity of ZntR. BSA was a negative control. Bio-T6SS4p: biotin-labelled T6SS4 promoter; Bio-T6SS4pM$_{zntR}$: biotin-labelled T6SS4 promoter replaced the ZntR binding region; T6SS4p: unlabelled T6SS4 promoter; URD: unrelated DNA fragment. (B) Putative ZntR binding region in T6SS4 promoter. The ATG initiation codon of the divergent gene upstream T6SS4 was highlighted in bold. The framed sequence in bold is correspond to putative ZntR binding region. putative -35 and -10 elements of the T6SS4 promoter are boxed, +1 denotes the transcription start point. The underlined sequences are the primers used in EMSA. (C) *β*-Galactosidase activity of the T6SS4 or T6SS4pM$_{zntR}$ promoter in *Y. pseudotuberculosis* wild-type or Δ*zntR* mutant. Data shown are the average of three independent experiments. The error bars indicate standard deviations of the mean. ***, P<0.001; n.s., not significant.

wild-type strain was more sensitive than the *zntR* mutant was (Fig. 4D). T6SS4 expression was reduced at high Zn^{2+} concentrations to prevent damage caused by excessive Zn^{2+} uptake. On the other hand, T6SS4 was up-regulated via ZntR and other regulators in the absence of Zn^{2+} in order to obtain sufficient Zn^{2+} to maintain cellular homeostasis.

T6SS4 and ZntR are essential for *Y. pseudotuberculosis* survival under oxidative stress

Metal ion homeostasis regulates the cellular level of ROS (Cornelis *et al.*, 2011). Zinc is an important metal ion in the Fenton reaction to reduce ROS levels (Faulkner and Helmann, 2011). High ROS levels induce damage to biological macromolecules, including proteins, lipids, and deoxyribonucleic acid, which can lead to cell death (Cornelis *et al.*, 2011). To investigate whether ZntR and T6SS4 are involved in ROS homeostasis, survival rates and the ROS levels in cells treated with hydrogen peroxide (H_2O_2) were measured. After incubation with H_2O_2, about 42% of the wild-type cells survived. The survival rates of a mutant lacking *clpV4*, the core component of T6SS4, and *zntR* were 15 and 25%, respectively, and a 16% survival rate was observed for the double mutant Δ*zntR*Δ*clpV4*. Expression of *clpV4* in the Δ*zntR*Δ*clpV4*

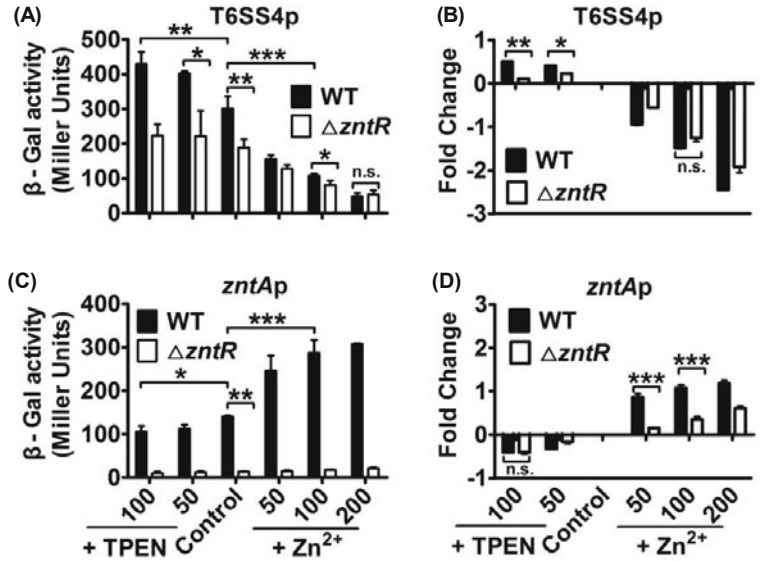

Fig. 4. T6SS4 and *zntA* expression are response to zinc conditions through ZntR-dependent mechanism. (A & C) Wild-type and Δ*zntR* mutant strains harbouring T6SS4*p*::*lacZ* or *zntAp*::*lacZ* were grown in the indicated amounts of Zn^{2+} or TPEN, and the expression of the reporter was measured. Data shown are the average of three independent experiments. (B & D) Fold change of the promoter activities were calculated by the equation: log_2 (The indicated strains/Control). The error bars indicate standard deviations of the mean. ***, P<0.001; **, P<0.01; *, P<0.05; n.s., not significant.

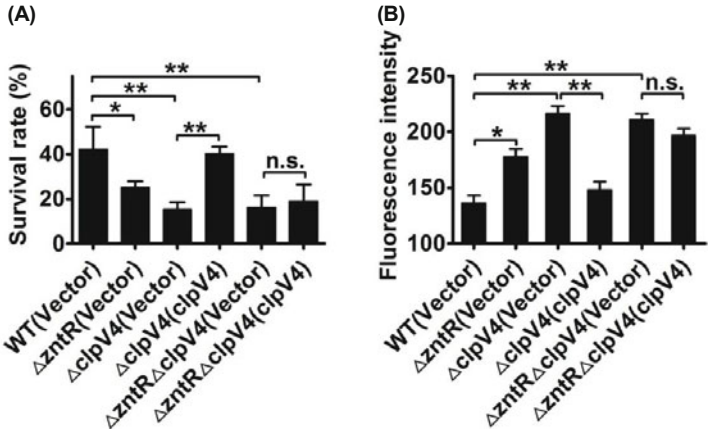

Fig. 5. T6SS4 and ZntR are essential for the survival of *Y. pseudotuberculosis* under oxidative stress. (A) Survival rate of oxidative stress was determined by measuring CFU/ml after treatment with H_2O_2 for 90 min. (B) Oxidative stress induced the generation of intracellular ROS. Intracellular ROS in mid-exponential phase bacteria exposed to H_2O_2 were stained with CM-H_2DCFDA dye (Invitrogen), fluorescence signals were measured using a SpectraMax M2 Plate Reader (Molecular Devices) with excitation/emission wavelengths of 495/520. Data shown are the average of three independent experiments and error bars indicate the standard deviation of the mean. **, P<0.01; *, P<0.05; n.s., not significant.

double mutant only slightly increased the survival rate, to 17% (Fig. 5A). The ROS level was also examined under oxidative stress using CM-H_2DCFDA fluorescent dye. These data showed that the ROS level in the Δ*zntR* and Δ*clpV4* single mutants and the Δ*zntR*Δ*clpV4* double mutant were all higher than in the wild-type strain. A complemented strain containing the pKT100-*clpV4* plasmid did not exhibit a reduced ROS level (Fig. 5B). These data suggest that T6SS4 plays a major role in ZntR-regulated oxidative survival by reducing the ROS level, and that the functions of T6SS4 and ZntR are interdependent.

Discussion

T6SS genes have been identified in more than 25% of bacteria with sequenced genomes, and many bacteria harbor more than one T6SS gene cluster. These different T6SSs are likely involved in different functions in the bacterial life cycle and regulated by different regulators (Bingle *et al.*, 2008; Boyer *et al.*, 2009). Here, in *Y. pseudotuberculosis*, we found that ZntR positively regulates T6SS4 by directly binding to the promoter region. The expression of T6SS4 is regulated by zinc concentration via ZntR to achieve zinc homeostasis and repress the ROS level, thereby preventing cell death under oxidative stress.

In *Y. pseudotuberculosis*, four T6SSs have been identified, and they are differentially thermo-regulated. T6SS4 is preferentially expressed at 26°C. Its expression is growth phase-dependent and subject to quorum sensing regulation, which is the first glance of T6SS4 regulation in *Y. pseudotuberculosis* (Zhang *et al.*, 2011). T6SS4 is involved in hydrogen ion export and pH homeostasis under acid stress, and T6SS4 expression is regulated by OmpR, the response regulator in the two-component system EnvZ-OmpR (Gueguen *et al.*, 2013; Zhang *et al.*, 2013). In addition, T6SS4 is positively controlled by RovM, which assists in bacterial survival under acidic and nutrient-limited environments (Song *et al.*, 2015). Recent studies have shown that RpoS, the stationary growth phase stress σ factor, and OxyR, a global oxidative stress regulator, directly regulate T6SS4 expression, which enhances bacterial survival in harsh environments and during interactions with hosts (Guan *et al.*, 2015; Wang *et al.*, 2015). Moreover, T6SS4 is cross regulated by multiple different regulators based on environmental factors and the host's immune system. The present study demonstrates that the zinc regulator ZntR directly regulates T6SS4 expression to maintain ROS homeostasis in *Y. pseudotuberculosis*.

Zn^{2+} is a trace metal ion essential for the growth of most organisms; however, too little zinc does not support growth, while excess zinc is toxic to cells (Faulkner and Helmann, 2011). As the level of zinc must be tightly regulated, cells maintain zinc homeostasis using exporter proteins and transport systems. Zur, a regulatory protein belonging to the Fur family, is the regulator of ZnuABC and ZupT (Patzer and Hantke, 1998; Grass *et al.*, 2002). On the other hand, ZntR, a member of the MerR family, is a metal-responsive transcriptional regulator responsible for the regulation of ZntA, ZntB, and ZitB (Khan *et al.*, 2002; Parsons and Heffron, 2005; Wang *et al.*, 2012). We found that ZntR positively regulates T6SS4 for zinc uptake and homeostasis, and this is a new regulatory target of ZntR. Through an analysis of the promoter sequences of T6SS4 and *zntA*, we found the ZntR-binding region upstream of the -35 and -10 elements in the T6SS4 promoter (Fig. 3B), but between the -35 and -10 elements in the *zntA* promoter (Brown *et al.*, 2003; Song *et al.*, 2015). The differences in the positions of the ZntR-binding region affect the regulation of two genes/gene clusters, a dual regulatory function for zinc homeostasis that provides a new mode of ZntR regulation.

The results of this study demonstrate that T6SS4, a zinc uptake secretion system, is positively regulated by ZntR to stabilize the intracellular zinc concentration and decrease the ROS level to prevent death under oxidative stress in *Y. pseudotuberculosis*. These results provide a new perspective on T6SS and ZntR, and they help explain the relationship between zinc import and export systems and the mechanism of zinc transport by T6SS. This mechanism increased the environmental adaptability of the bacterium under zinc-limited conditions or oxidative stress.

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Conflict of Interests

The authors declare that they have no competing interests.

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