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The stringent response factor, RelA, positively regulates T6SS4 expression through the RovM/RovA pathway in *Yersinia pseudotuberculosis*

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ABSTRACT

The type VI secretion system (T6SS) is a versatile molecular machinery widely distributed in Gram-negative bacteria. The activity of the T6SS is tightly regulated by various mechanisms, including quorum sensing (QS), iron concentration, and transcriptional regulators. Here we demonstrated that the stringent response regulator, RelA, contributes to bacterial resistance to multiple environmental stresses in *Yersinia pseudotuberculosis*. We also revealed that the stress resistance function of stringent response (SR) was partially mediated by the general stress response T6SS4 system. RelA positively regulates the expression of T6SS4 to combat various stresses in response to nutrition starvation collectively mediated by the RovM and RovA regulators. These findings revealed not only the important role of T6SS4 in SR induced stress resistance, but also a new pathway to regulate T6SS4 expression in response to starvation stress.

1. Introduction

The type VI secretion system (T6SS) is a versatile bacterial protein secretion machinery widely distributed in one quarter of the sequenced Gram-negative bacterial genomes (Ho et al., 2014; Russell et al., 2014; Hachani et al., 2016). The best-characterized function of T6SSs is delivery of antibacterial toxins, such as nucleases, cell wall-degrading enzymes, and membrane-targeting enzymes, into prokaryotic cells to facilitate inter-species competition (Russell et al., 2011; Dong et al., 2013; Russell et al., 2013; Ma et al., 2014; Yang et al., 2018). Some T6SSs associated with pathogens are necessary for full virulence by injecting effectors into target eukaryotic cells to modulate host immunity and inflammation (Aubert et al., 2016; Jiang et al., 2016). Recently, several T6SSs have also been reported to be involved in acquisition of essential micronutrients such as iron, manganese and zinc (Wang et al., 2015; Lin et al., 2017; Si et al., 2017a, 2017b). In addition, accumulating data indicate that T6SS is involved in stress resistance in various bacteria (Weber et al., 2009; Goldova et al., 2011; Gueguen et al., 2013; Zhang et al., 2013; Song et al., 2015; Wang et al., 2015).

Whereas many bacteria possess one to two T6SS gene clusters, the enteric pathogen *Yersinia pseudotuberculosis* possesses four T6SS gene clusters which are believed to confer distinct functions for specific niches in the lifecycle of the bacterium (Zhang et al., 2011; Yang et al., 2018). Among the four T6SS clusters, T6SS4 was reported to be essential for bacterial survival under oxidative, acidic, heat and osmotic stress and for resistance to deoxycholate (Gueguen et al., 2013; Zhang et al., 2013; Wang et al., 2017). Accordingly, the expression of T6SS4 is regulated by various stress response regulators such as the stationary growth phase stress σ factor RpoS (Guan et al., 2015), the global oxidative stress regulator OxyR (Wang et al., 2015), and the osmotic regulator OmpR (Gueguen et al., 2013; Zhang et al., 2013). In addition, the expression of T6SS4 is nutrient status-dependently regulated by a LysR-type regulator, RovM (Song et al., 2015). RovM is a nutrient dependent regulator and it's expression responds to the availability of nutrients, regulated by the carbon storage regulator (Csr) system, including the RNA-binding protein CsrA and the two regulatory RNAs CsrB and CsrC (Heroven et al., 2008). As a central regulator of the Crp-CsrABC-RovM regulatory cascade, RovM conversely regulates the expression of T6SS4 and AR3 acid resistance systems in response to the availability of nutrients, possibly mediated by CsrABC (Song et al., 2015).

Similar to the CsrABC system, the stringent response (SR) defines

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another global regulatory network of bacteria for sensing nutrient availability (Edwards et al., 2011). The stringent response is triggered by the deprivation of intracellular amino acids and is characterized by upregulation of genes required for amino acid biosynthesis, and downregulation of genes associated with cell growth (Potrykus and Cashel, 2008; Hauryliuk et al., 2015). The core molecules of stringent response are the nucleotide secondary messenger guanosine tetraphosphate and guanosine pentaphosphate (collectively referred to as (p)ppGpp), which help the bacteria adapt to starving conditions chiefly via binding to RNA polymerase to affect transcription (Potrykus and Cashel, 2008; Hauryliuk et al., 2015). As the main bacterial ppGpp synthetase, RelA produces ppGpp in response to the presence of uncharged tRNA in the ribosomal A-site. While the stringent response was originally identified as an adaptation to amino acid starvation, it was also reported to play a broad role in bacterial adaptation to environmental stress (Okada et al., 2002; Yan et al., 2009; Vercruysse et al., 2011; Gaca et al., 2013; Khakimova et al., 2013; Hauryliuk et al., 2015) and virulence (Dahl et al., 2003; Nakanishi et al., 2006; Hesketh et al., 2007). However, although our understanding of the stringent response is becoming deeper, the mechanisms underlying stress resistance of stringent response remain largely unknown.

We report here that the stringent response regulator, RelA, activates the expression of the stress-resistant T6SS4 in *Y. pseudotuberculosis* mediated by the global transcriptional regulators RovM/RovA collaborately. We reveal that the stress resistance function of stringent response was partially mediated by the stress-resistant T6SS4.

2. Methods and materials

2.1. Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in Table S1. *Escherichia coli* were cultured in Luria-Bertani (LB) medium at 37 °C with appropriate antibiotics. *Y. pseudotuberculosis* strains were cultured in Yersinia-Luria-Bertani (YLB) broth (0.5% NaCl, 1% tryptone, 0.5% yeast extract, pH 7.0), or M9 minimal medium (Na_2HPO_4, 6 g/L; KH_2PO_4, 3 g/L; NaCl, 0.5 g/L; NH_4Cl, 1 g/L; $MgSO_4$, 1 mM; $CaCl_2$, 0.1 mM; glucose 0.2%, pH 7.0) at 26 °C with appropriate antibiotics when necessary. The *Y. pseudotuberculosis* strain YPIII was the parent of all derivatives used in this study. In-frame deletions were generated as described previously (Xu et al., 2014). Cellular growth was monitored based on the optical density (OD) at 600 nm. Antibiotics were added at the following concentrations: nalidixic acid, 20 μg/mL; ampicillin, 100 μg/mL; kanamycin, 50 μg/mL; chloramphenicol, 20 μg/mL.

2.2. Plasmid construction

Primers used in this study were listed in Table S2. The *lacZ* fusion reporter vector pDM4-P_{T6SS4}::*lacZ* was made previously (Zhang et al., 2011). The reporter vector pDM4-P_{rovM}::*lacZ* and pDM4-P_{rovA}::*lacZ* was constructed in a similar manner using primers P_{rovM}-F-SalI/P_{rovM}-R-XbaI and P_{rovA}-F-SalI/P_{rovA}-R-XbaI, respectively. The plasmid pDM4-Δ*rovM* (*ypk_1559*) was made in our previous work (Song et al., 2015). The knockout plasmid pDM4-Δ*relA* (*ypk_3449*) and pDM4-Δ*rovA* (*ypk_1876*) were constructed in a similar manner using primer pairs *relA*-1F-SalI/*relA*-1R, *relA*-2 F/*relA*-2R-BglII and *rovA*-1F-SalI/*rovA*-1R, *rovA*-2 F/*rovA*-2R-BglII, respectively. The complementation plasmids pKT100-*relA* and pKT100-*rovA* were constructed using the primers *relAcom*-F-BamHI/*relA*com-R-SalI and *rovAcom*-F-BamHI/*rovA*com-R-SalI, respectively. To express His_6-tagged RovA, plasmid pET15b-*rovA* was constructed. Briefly, primers *rovA*-F-NdeI and *rovA*-R-BamHI were used to PCR amplify the *rovA* gene fragment from the *Y. pseudotuberculosis* genome. The PCR products of *rovA* were digested with NdeI/BamHI and inserted into the NdeI/BamHI sites of pET15b to generate pET15b-*rovA*.

Site-directed mutagenesis of $RelA_{G251E}$ and $RelA_{H354Y}$ were carried out by overlap PCR. Briefly, DNA of mutant $relA_{G251E}$ and $relA_{H354Y}$ were amplified by two rounds of PCR. Take example of $relA_{G251E}$, primer pairs *relAcom*-F-BamHI/$relA_{G251E}$-M1R and $relA_{G251E}$-M2F/*relAcom*-R-SalI were used to amplify segments 1 and 2 respectively. The second round of PCR was carried out by using *relAcom*-F-BamHI/*relAcom*-R-SalI as primer pair, while fragment 1 and fragment 2 as templates to obtain the $relA_{G251E}$ fragment. The $relA_{G251E}$ DNA fragment was digested by BamHI/SalI and cloned into similar digested pKT100 to produce pKT100-$relA_{G251E}$. The pKT100-$relA_{H354Y}$ plasmid was constructed with similar methods.

2.3. Construction of chromosomal fusion reporter strains and β-galactosidase assays

The *lacZ* fusion reporter vectors pDM4-P_{T6SS4}::*lacZ*, pDM4-P_{rovM}::*lacZ* and pDM4-P_{rovA}::*lacZ* were transformed into *E. coli* S17-1λpir and mated with *Y. pseudotuberculosis* strains as described previously (Zhang et al., 2013). The *lacZ* fusion reporter strains were grown to stationary phase in YLB broth or M9 medium at pH 7.0 under 26 °C, and β-galactosidase activity was assayed using ONPG (o-nitrophenyl-β-D-galactopyranoside) as the substrate. These assays were performed in triplicate at least three times, and error bars represent standard deviations.

2.4. Survival assays

Mid-exponential phase *Y. pseudotuberculosis* strains grown in YLB medium were collected, washed, and diluted 50-fold into M9 medium containing NaCl (0.5 M), or HCl (pH 4.5) and incubated at 26 °C for 1 h, or 1.5 mM H_2O_2 for 30 min. After treatment, the cultures were serially diluted 1000-fold and plated onto YLB agar plates, and 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 (Si et al., 2014; Wang et al., 2015). The assays were performed in triplicate at least three times, and error bars represent standard deviation.

2.5. Fluorescence dye-based intracellular ROS detection

To detect intracellular total ROS, the fluorescent reporter dye 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetylester (CM-H_2DCFDA, Life Technologies) was used as previously described (Si et al., 2014; Dong et al., 2015). Briefly, 1 ml bacterial samples (OD_{600} = 1.6) were collected and resuspended in 1 ml PBS with 5 mM H_2O_2, treated for 30 min at 26 °C, 100 rpm. After washed twice, the samples were resuspended in 1 ml of PBS containing 10 μM CM-H_2DCFDA and incubated in dark for 20 min. The cells were then pelleted, the supernatant removed, and were resuspended in 1 ml filter-sterilized PBS. Two hundred microliters of the resultant cell suspension were transferred to a black 96-well plate in dark. Fluorescence signals were measured using a SpectraMax M2 Plate Reader (Molecular Devices) with excitation/emission wavelengths of 502/521 nm. The results shown represented the mean of one representative assay performed in triplicate, and error bars represent standard deviations.

2.6. Transcriptomics analysis

RNA-Seq-based comparative transcriptomics analysis of WT and Δ*relA* mutant was performed to detect the differentially expressed genes (DEGs). Single colonies of two strains were picked and cultured in YLB medium till stationary phase, after then bacteria were transferred to M9 medium by 1:100 and grown to logarithmic period (OD_{600} = 0.5). The bacteria were harvested for RNA extraction and cDNA synthesis. The transcriptomics sequencing was performed by BGI-tech (Shenzhen, China). Based on the alignment with reference gene and genome file in KEGG database, we got the expression level of the two bacteria strains, showing by RPKM (Reads per kilobase transcriptome per million

mapped reads). The fold changes of DEGs between Δ*relA* mutant and WT strain were calculated according to $\log_2$(RPKM of Δ*relA*/WT).

2.7. Quantitative real time (qRT)-PCR analysis

Total RNA was isolated from exponentially growing *Y. pseudotuberculosis* strains using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA was converted to cDNA using M-MLV Reverse Transcriptase (TaKaRa, Dalian, China) following the manufacturer's instructions. qRT-PCR analysis was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) with 20 μl volume of PCR mixture containing 10 μl 2 × TransStart green qPCR supermix (TransGen, China), 0.5 μl each primer and l μl cDNA. After 15 s of denaturation at 95 °C, 40 cycles of 95 °C for 15 s and 55 °C for 30 s were performed. The qRT-PCR primers were listed in Table S2. To standardise the results, the relative abundance of 16S rRNA was used as an internal standard and each reaction was performed in triplicate. The expression of target genes was calculated as relative fold values using the $2^{-\triangle\triangle CT}$ method. These assays were performed in triplicate at least three times, and error bars represent standard deviations of the mean.

2.8. Electrophoretic mobility shift assay (EMSA)

DNA probes (279 bp T6SS4 promoter fragments) were amplified from the T6SS4 promoter region using primers P_{T6SS4}-F/P_{T6SS4}-R. As negative control, a 300 bp fragment amplified from *ypk_3566* (*impA*) coding region using primers control-F/control-R was included in the binding assay. Increasing concentrations of purified His_6-RovA (0.75, 1.5 and 3 μM) were incubated with 20 nM DNA probes in EMSA buffer (20 mM Tris-HCl, pH 7.4, 4 mM MgCl2, 100 mM NaCl, 1 mM dithiothreitol, 10% glycerol). After incubation for 30 min at room temperature, the binding reaction mixture was subjected to electrophoresis on a 6% native polyacrylamide gel containing 5% glycerol in 0.5 × TBE (Tris-borate-EDTA) electrophoresis buffer, and the DNA probe was detected using SYBR Green (Zhang et al., 2013; Si et al., 2015).

2.9. DNase I footprinting assay

DNase I footprinting assays were performed according to (Wang et al., 2015) with minor modifications. Briefly, the promoter region of T6SS4 was PCR amplified with Dpx DNA polymerase (TOLO Biotech, China) using primer pair P_{T6SS4}-F/P_{T6SS4}-R, and the fragment was cloned into the pMD-18 T vector (TaKaRa), which was further used as a template for the preparation of fluorescent FAM-labelled probes with primers M13R(FAM labelled) and M13 F(−47). The FAM-labelled probes were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, USA) and quantified using the NanoDrop 2000C (Thermo, USA). For the DNase I footprinting assay, 400 ng probes was incubated with different amounts of His_6-RovA in a total volume of 40 μl in the same buffer. After incubation for 30 min at 30 °C, 10 μl solution containing about 0.010 U DNase I (Promega, USA), and 100 nmol freshly prepared $CaCl_2$ was added and further incubated for 1 min at 25 °C. The reaction was stopped by adding 140 μl DNase I stop solution (200 mM unbuffered sodium acetate, 30 mM ethylenediaminetetraacetic acid and 0.15% SDS). Samples were then extracted using phenol/chloroform, precipitated with ethanol and the pellets were dissolved in 35 μl MiliQ water. The preparation of the DNA ladder, electrophoresis and data analysis was the same as described previously (Wang et al., 2012), except that the GeneScan-LIZ500 size standard (Applied Biosystems) was used.

2.10. Western blot analysis

Western blot analysis was performed as described previously (Xu et al., 2014). Briefly, samples were resolved by SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Millipore). The membrane was blocked in 5% (w/v) non-fat milk for 4 h at room temperature and incubated with primary antibodies at 4 °C overnight: anti-VSVG (Santa Cruz Biotechnology, USA), 1:500; anti-RNA pol β (Santa Cruz Biotechnology, USA), 1:500. Then the membrane was washed three times in TBST buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.4) and incubated with a 1:5000 dilution of horseradish peroxidase-conjugated secondary antibodies (Shanghai Genomics) for 1 h. Signals were detected using the ECL plus kit (GE Healthcare) following the manufacturer's protocol.

2.11. Statistical analysis

Statistical analyses were performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Survival assay, ROS determination, LacZ activity, and gene expression data were performed using an analysis of variance with a subsequent multiple comparison by using paired two-tailed Student's *t*-test.

3. Results

3.1. RelA contributes to bacterial resistance to multiple environmental stresses

As the stringent response factor, RelA was reported to be involved in the resistance to multiple environmental stresses such as oxidative stress, osmotic stress and antibiotics (Okada et al., 2002; Yan et al., 2009; Khakimova et al., 2013). Similarly, the Δ*relA* mutant showed decreased resistance to osmotic, acid, and H_2O_2 stresses compared to the *Y. pseudotuberculosis* wild-type, and complementation with the pKT100-*relA* plasmid restored the stress resistance to wild-type level (Fig. 1A-C). Note that deletion of the *relA* gene did not affect the growth rate of *Y. pseudotuberculosis* under nutrient-rich conditions (Data not shown). Moreover, the Δ*relA* mutant exhibited increased intracellular ROS levels, which was restored to the wild-type level by providing the pKT100-*relA* plasmid (Fig. 1D). These results confirm that RelA contributes to bacterial resistance to multiple environmental stresses in *Y. pseudotuberculosis*.

3.2. Genome-wide analysis of genes regulated by RelA in Y. pseudotuberculosis

To systematically identify genes regulated by RelA, we performed RNA sequencing (RNA-seq)-based comparative transcriptomic analysis of the *Y. pseudotuberculosis* wild-type and the Δ*relA* mutant. Bacteria in the mid-logarithmic growth phase were harvested for RNA extraction and transcriptomic sequencing. The expression of a total of 712 genes was up- or down-regulated greater than 1.0-fold (Table S3). The transcriptomic data were verified by qRT-PCR analysis of 10 random chosen genes. As shown in Fig. 2A, the $\log_2$-transformed mean values of 3 biological replicates for each gene were in good consistency with the $\log_2$-transformed fold changes observed in the RNA-seq data (Fig. 2A). The functions of the differentially expressed genes (DEGs) were identified using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis, and the major pathways regulated by RelA were summarized. As shown in Fig. 2B, the DEGs were involved in multiple KEGG pathways, including those related to amino acids and nucleotide biosynthesis, lipid metabolism, cell motility, bacterial secretion systems, transport activities, transcription and translation processes, and responses to stress. Bacteria that undergo the SR in response to stresses (including starvation) have lower stable RNA, ribosomal protein, and DNA replication rates (Traxler et al., 2008). Similarly, we found the ribosomal protein-associated genes (*ypk_0283-0299*) and purine metabolism genes were upregulated in the Δ*relA* mutant, reflecting negative regulation by RelA. Moreover, genes encoding ATP-binding cassette (ABC) transporters for carbohydrate uptake (*ypk_0431*) and amino acid transport (*ypk_0377-0378, 1094-1096*) were upregulated. Interestingly,

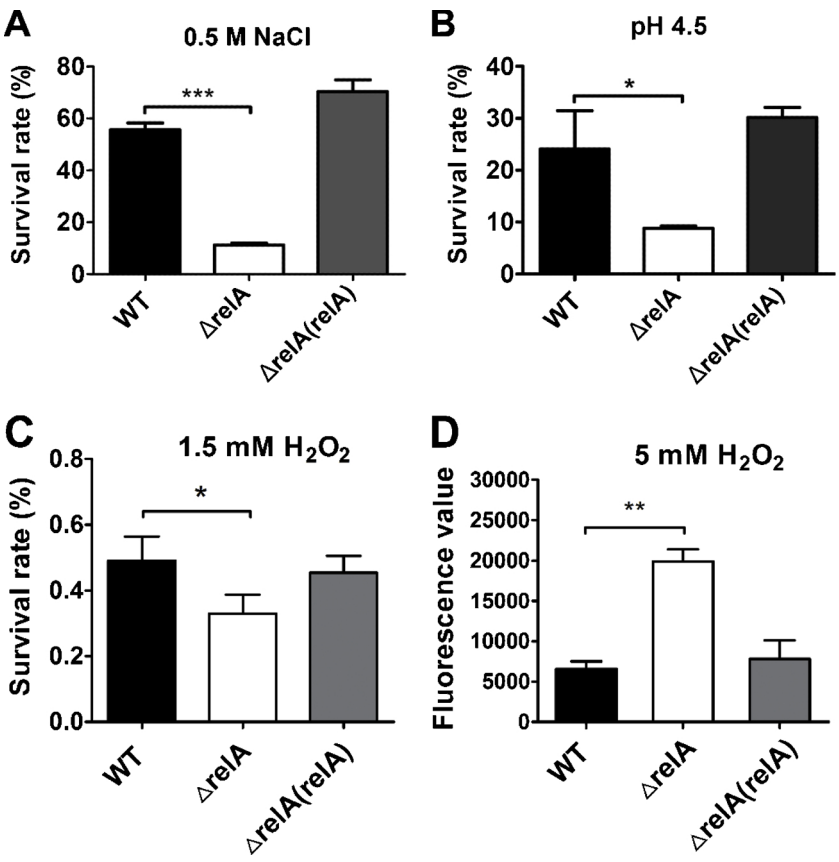

Fig. 1. The role of RelA in stress resistance. (A–C) Survival rates of *Y. pseudotuberculosis* wild-type, Δ*relA* mutant, and the complemented strain Δ*relA(relA)* after challenge with 0.5 M NaCl (**A**) or pH4.5 (**B**) for 60 min, or with H_2O_2 (1.5 mM) for 30 min (**C**) were determined. **(D)** The ROS levels in the *Y. pseudotuberculosis* wild-type, Δ*relA* mutant, and the complemented strain Δ*relA(relA)* were measured by using the CM-H_2DCFDA fluorescence determination assay after exposure to H_2O_2 (5 mM) for 30 min at 26 °C. Data represent the mean ± SD of three biological replicates, each of which was performed with three technical replicates. ***: $p < 0.001$, **: $p < 0.01$, *: $p < 0.05$.

the response regulator RpoS (*ypk_2078*) and a glycosidase-encoding gene (*ypk_0379*) were also upregulated, indicating increased sensitivity to environmental factors. In contrast, genes related to the cold-shock response (*ypk_0442-0444*) were downregulated in Δ*relA*, as were genes encoding flagellum components, suggesting a lower resistance to cold stress and reduced motility. Moreover, the expression level of type VI secretion system-related genes (*ypk_0383-0401* and *ypk_3549-3566*) was also downregulated in Δ*relA*, which indicated the dysfunction related to T6SS.

3.3. RelA positively regulates T6SS4 expression in Y. pseudotuberculosis

T6SS is a widely distributed bacterial protein export apparatus found in one quarter of Gram-negative bacteria (Ho et al., 2014; Russell et al., 2014; Hachani et al., 2016). Our transcriptome data revealed that most of the genes in the T6SS4 operon (*ypk_3549-3566*) were down-regulated to below 10% in the Δ*relA* mutant compare to the wild-type strain, except for *ypk_3549* (13.4%) and *ypk_3563* (40.59%) (Table 1). For example, VgrG, which forms the spike complex for the T6SS needle structure, was down-regulated to 3.69%. IcmF, a periplasmic domain protein, was down-regulated to 5.3%. Similar results were observed for other T6SS4 component genes. These data suggest that T6SS4 is positively regulated by RelA.

To further investigate the role of RelA in *T6SS4* expression, we introduced a single copy of the transcriptional fusion *T6SS4p::lacZ* into the chromosomes of *Y. pseudotuberculosis* wild-type, Δ*relA* and the complementary strain. The activity of LacZ was quantified as described (Miller, 1992). As shown in Fig. 3A, the β-galactosidase activity in the Δ*relA* mutant decreased to 26.2% compared with the wild-type; moreover, the decrease was almost completely restored to the wild-type level by introducing a plasmid expressing RelA (pKT100-*relA*) into the Δ*relA* mutant. qRT-PCR also showed that the expression of T6SS4 component genes *clpV4* (*ypk_3559*) and *hcp4* (*ypk_3563*) required RelA (Fig. 3B). In addition, deletion of *relA* resulted in a significant decrease in Hcp4-VSVG secretion (Fig. 3C), further supporting the regulating effect of RelA on T6SS4.

The main function of RelA is to synthesize (p)ppGpp. The function of RelA in (p)ppGpp synthesis is essential for activating T6SS4 expression, as complementation with $relA_{G251E}$ and $relA_{H354Y}$, two (p)ppGpp null mutants, failed to recover the T6SS4 promoter activity in the Δ*relA* mutant (Fig. 3A). These results demonstrate that RelA positively regulates T6SS4 expression by catalyzing the synthesis of (p)ppGpp.

3.4. RelA activates RovM expression

Because RovM, a LysR-type regulatory protein, activates T6SS4 expression by directly binding to the T6SS4 promoter (Song et al., 2015), and the expression of RovM is in response to the availability of nutrients (Heroven et al., 2008), we speculated that RelA may regulate T6SS4 expression by the mediation of RovM. To test this hypothesis, we first investigated the role of RelA in RovM expression by chromosomal P_{rovM}::*lacZ* fusion reporter analysis. As shown in Fig. 4A, the activity of the *rovM* promoter in Δ*relA* was significantly lower than that in the wild-type, and the promoter activity was restored to the wild-type level by introducing the complementary plasmid pKT100-*relA*. However, complementation with (p)ppGpp null mutants $relA_{G251E}$ and $relA_{H354Y}$ failed to restore the *rovM* promoter activity. The reduced expression of *rovM* in Δ*relA* was also confirmed by qRT-PCR analysis (Fig. 4B). These results indicate that the nutrient-dependent expression of RovM is dependent on RelA.

To determine whether the regulation of RelA on T6SS4 was mediated by RovM, we constructed a Δ*relA*Δ*rovM* double mutant. Unexpectedly, introducing the complementary plasmid pKT100-*relA* to the Δ*relA*Δ*rovM* double mutant still fully restored the T6SS4 promoter activity to the wild-type level as in the Δ*relA* single mutant (Fig. 4C). That RelA can regulate T6SS4 expression in the absence of RovM

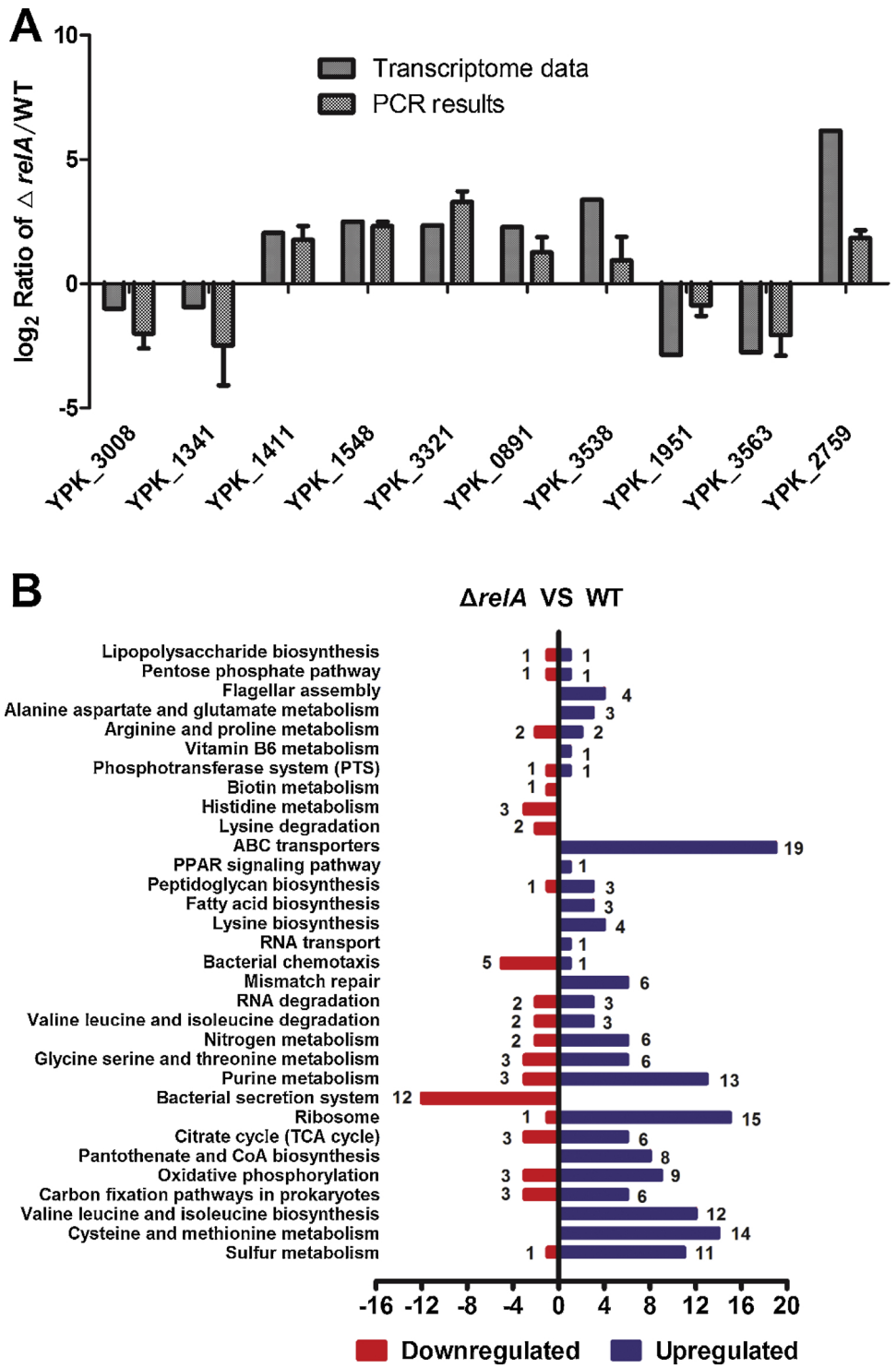

Fig. 2. Transcriptomics analysis of RelA regulated genes in *Y. pseudotuberculosis*. **(A)** 10 representative genes were evaluated for validation of the RNA-seq data using qRT-PCR. **(B)** KEGG pathway analysis of differentially expressed genes (Δ*relA* mutant VS wild-type). The red and blue bars represent down- and up-regulated genes, respectively, and the numeric labels represent the number of genes related to that pathway.

suggesting that the RelA-regulated T6SS4 expression was also mediated by other regulators besides RovM.

3.5. RovA directly activates T6SS4 expression

RovA, a transcriptional activator of the SlyA/Hor family repressed by RovM, was previously reported to regulate T6SS4 expression in *Y. pestis* (Cathelyn et al., 2006). To investigate whether RovA is one of the other regulators that mediate the RelA-regulated T6SS4 expression, we examined the role of RovA in T6SS4 expression in *Y. pseudotuberculosis*. T6SS4 expression was determined by chromosomal P_{T6SS4}::*lacZ* fusion reporter analysis. As shown in Fig. 5A, deletion of *rovA* moderately but significantly reduced the activity of the T6SS4 promoter, which was fully restored by introducing a complementary plasmid expressing RovA (pKT100-*rovA*).

Next, EMSA assay was performed to investigate whether RovA

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

Gene ID	Product	Description	$\log_2$(Δ*relA*/WT)	Percentile	Probability
ypk_3549		Conserved hypothetical protein	−2.8994	13.40	0.9458
ypk_3550	IcmF	Type VI secretion system protein ImpL	−3.3809	9.60	0.9199
ypk_3551	ImpK	Type VI secretion system protein ImpK	−4.0827	5.90	0.9459
ypk_3552	ImpL	Type VI secretion system protein ImpJ	−4.6288	4.04	0.9496
ypk_3553	VasD	type VI secretion system protein VasD	−4.9940	3.14	0.9490
ypk_3554		Conserved hypothetical protein	−4.6565	3.97	0.9588
ypk_3555		Conserved hypothetical protein	−4.7694	3.67	0.9543
ypk_3556		Pentapeptide repeat protein	−4.0070	6.22	0.9370
ypk_3557		Pentapeptide repeat protein	−4.0874	5.88	0.9247
ypk_3558	VgrG	T6SS secreted protein VgrG	−4.7616	3.69	0.9578
ypk_3559	VasG	Type VI secretion system protein VasG	−4.8447	3.48	0.9419
ypk_3560	ImpH	Type VI secretion system protein ImpH	−3.8719	6.83	0.9341
ypk_3561	ImpG	Type VI secretion system protein ImpG	−4.7125	3.81	0.9407
ypk_3562	ImpF	Type VI secretion system protein ImpF	−4.2390	5.30	0.9377
ypk_3563	Hcp	T6SS secreted protein Hcp	−1.3009	40.59	0.9720
ypk_3564	ImpC	Type VI secretion system protein ImpC	−4.1080	5.80	0.9578
ypk_3565	TssB	Type VI secretion system protein TssB	−5.0273	3.07	0.9428
ypk_3566	TssC	Type VI secretion system protein TssC	−5.8827	1.69	0.9476

Percentile: (Δ*relA*/WT)*100%; Probability: the coefficient to evaluate differential expression.

regulates T6SS4 expression in a direct manner. Incubation of a probe harbouring the T6SS4 promoter (P_{T6SS4}) sequence (-133 to -411 relative to the ATG start codon of the first ORF of the T6SS4 operon) with His_6-RovA led to the formation of DNA-protein complexes, and the abundance of such complexes depended on the amount of RovA. However, no retarded mobility was observed when the control DNA fragments were added (Fig. 5B).

To further identify the precise RovA-binding site, DNase I footprinting analysis was performed (Fig. 5C). Two RovA-protected DNA regions extended from -320 to -229 bp and -156 to -98 bp upstream of the initiation codon of the first T6SS4 ORF were identified (Fig. 5D). In *Y. pseudotuberculosis*, RovA activated its own expression and that of the virulence factor invasin in response to a moderate growth temperature (Heroven et al., 2004). Both of the *inv* and *rovA* promoter regions contain two AT-rich RovA-binding sites located upstream of the -35 element of the promoters. Similar AT-rich RovA-binding sites located upstream of the -35 element, “TAAATTTAATAAAT” and “ATTATAAA ATATA”, were also identified from the two RovA-protected DNA regions in the T6SS4 promoter, respectively (Fig. 5D). Together, these results demonstrate that RovA positively regulates T6SS4 expression by recognizing two conserved AT-rich RovA-binding sites in the T6SS4 promoter.

3.6. Both RovM and RovA are required to mediate RelA-regulated T6SS4 expression

To investigate the role of RovA in the mediation of RelA-regulated T6SS4 expression, we constructed the Δ*relA*Δ*rovA* double mutant and the Δ*relA*Δ*rovA*Δ*rovM* triple mutant. While introducing the complementary plasmid pKT100-*relA* to the Δ*relA*Δ*rovA* double mutant still restored the T6SS4 promoter activity to the wild-type level, it failed to recover the promoter activity in the Δ*relA*Δ*rovA*Δ*rovM* triple mutant (Fig. 6A). These results suggest that the RelA-regulated T6SS4 expression was mediated by RovM and RovA together, and both regulators are necessary to mediate the regulation. This conclusion was further corroborated by overexpressing *relA* in different *Y. pseudotuberculosis* strains. As shown in Fig. 6B, while overexpression of *relA* greatly improved the T6SS4 promoter activities in the wild-type and even in the Δ*rovM* and Δ*rovA* single mutants, it failed to improve the T6SS4 promoter activity in the Δ*rovA*Δ*rovM* double mutant. Altogether, these data indicate that RelA functions upstream of RovM and RovA to regulate T6SS4 expression.

3.7. RovM suppresses the expression of RovA in Y. pseudotuberculosis

Disruption of *rovM* resulted in a significant increase in RovA and invasin production and enhanced internalization of *Y. pseudotuberculosis* into host cells (Heroven and Dersch, 2006; Heroven et al., 2008).

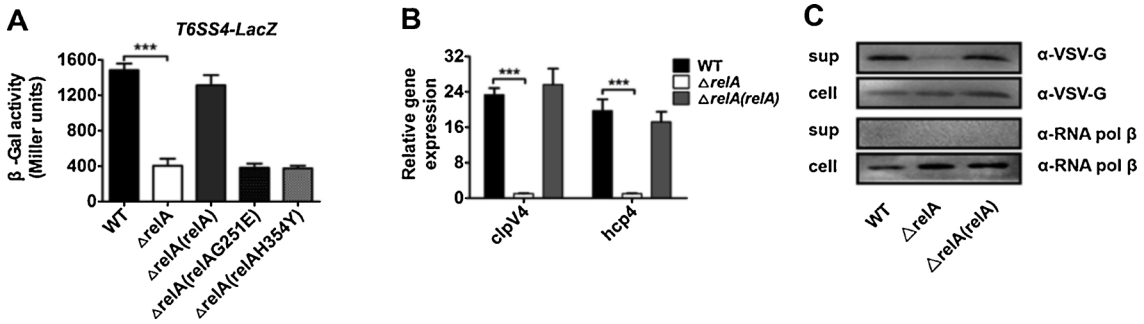

Fig. 3. RelA positively regulates T6SS4 expression. (A) β-galactosidase analyses of T6SS4 promoter activities in the *Y. pseudotuberculosis* wild-type, Δ*relA* mutant, and the complemented strain Δ*relA*(*relA*), Δ*relA*(*relAG251E*) and Δ*relA*(*relAH354Y*) grown to stationary phase in M9 medium. **(B)** qRT-PCR analysis of mRNA levels of *clpV* and *hcp* in indicated strains. Data represent the mean ± SD of three biological replicates, each of which was performed with three technical replicates. ***: $p < 0.001$. **(C)** The wild-type, Δ*relA* and its complemented strain Δ*relA*(*relA*) expressing C-terminal VSV-G-tagged Hcp4 were grown in M9 media to the logarithmic phase at 26 °C. Expression (cell) and secretion (sup) of Hcp4-VSV-G was detected by immunoblotting using anti-VSV-G antibodies. The loading control detected by anti-RNA pol-β was shown for total protein lysate.

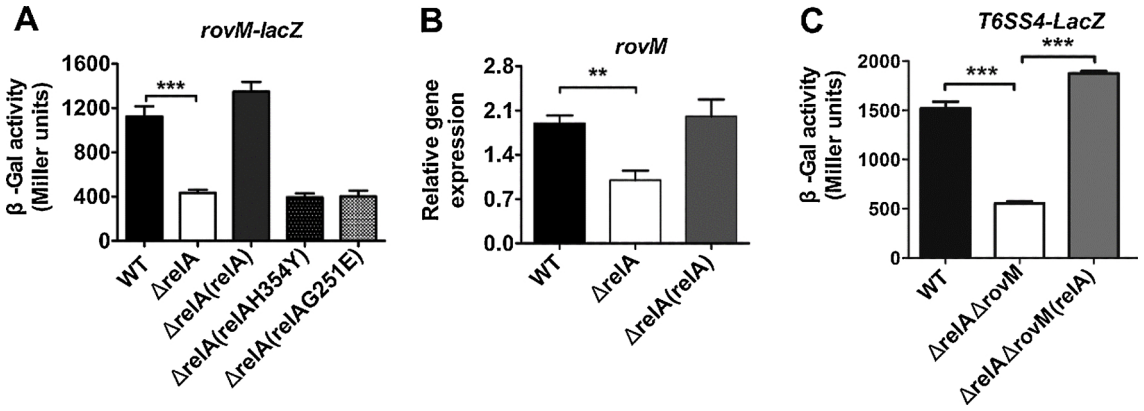

Fig. 4. RelA positively regulates RovM expression. (A) β-galactosidase analyses of *rovM* promoter activities in the *Y. pseudotuberculosis* wild-type, Δ*relA* mutant, and the complemented strain Δ*relA*(*relA*), Δ*relA(relAG251E)* and Δ*relA(relAH354Y)* grown to stationary phase in M9 medium. **(B)** qRT-PCR analysis of mRNA levels of *rovM* in the *Y. pseudotuberculosis* wild-type, Δ*relA* mutant, and the complemented strain Δ*relA*(*relA*). (**C**) β-galactosidase analyses of T6SS4 promoter activities in the *Y. pseudotuberculosis* wild-type, Δ*rovM*Δ*relA* mutant, and the complemented strains Δ*rovM*Δ*relA(relA)*. Data represent the mean ± SD of three biological replicates, each of which was performed with three technical replicates. ***, $p < 0.001$, **, $p < 0.01$.

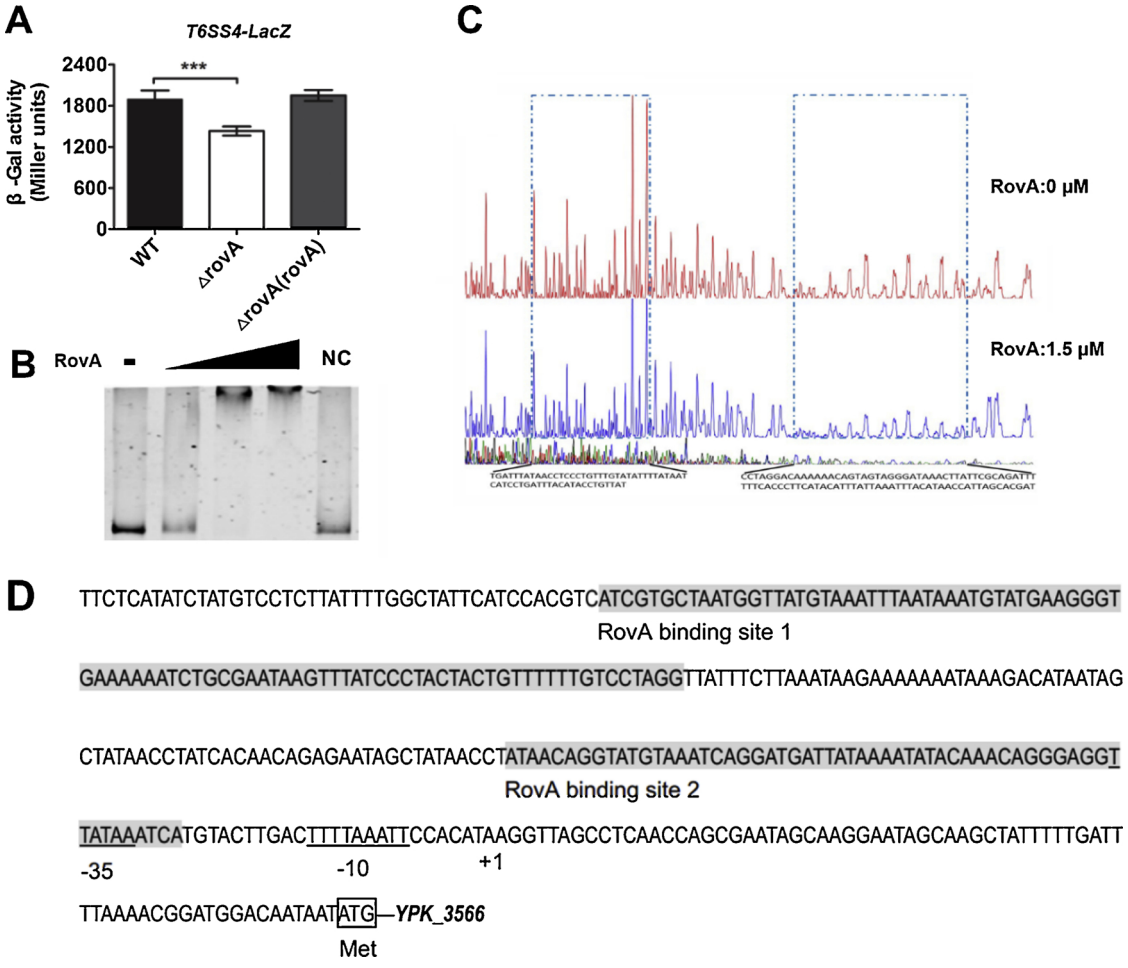

Fig. 5. RovA directly regulates T6SS4 expression. (**A**) β-galactosidase analyses of T6SS4 promoter activities in the *Y. pseudotuberculosis* wild-type, Δ*rovA* mutant, and the complemented strain Δ*rovA(rovA)*. Data represent the mean ± SD of three biological replicates, each of which was performed with three technical replicates. ***: $p < 0.001$. (**B**) EMSA was performed to analyse the interactions between His_6-RovA and the T6SS4 promoter (P_{T6SS4}). Increasing amounts of RovA (0.75, 1.5 and 3 μM) and 20 nM DNA fragment were used. (**C**) Identification of the RovA-binding site within the T6SS4 promoter using the DNase I footprinting assay. (**D**) The RovA binding sites detected in (**C**) were shown in the T6SS4 promoter region. β-galactosidase analyses of T6SS4 promoter activities in the *Y. pseudotuberculosis* wild-type, Δ*rovA*Δ*relA* mutant, and the complemented strains Δ*rovA*Δ*relA(relA)*, Δ*rovA*Δ*relA(rovA)*.

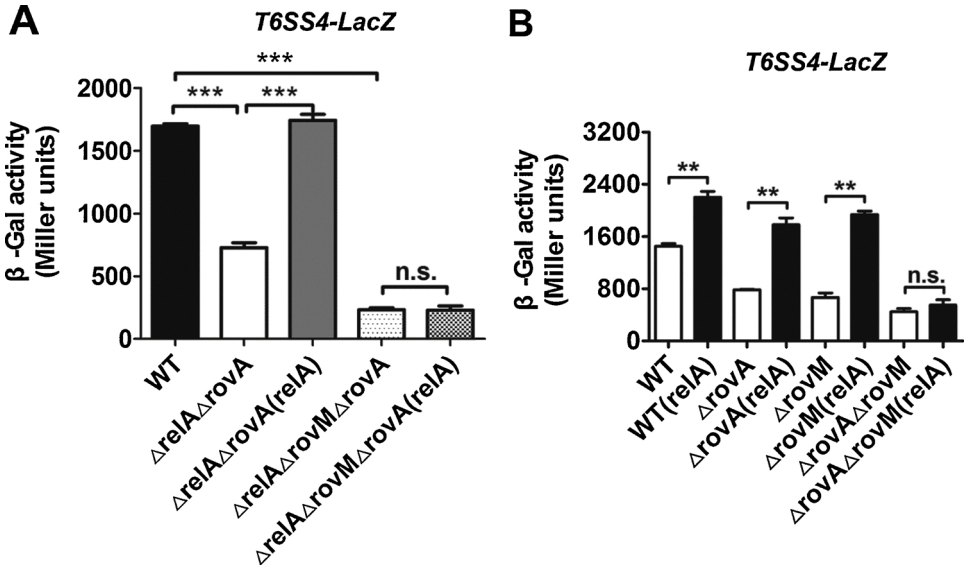

Fig. 6. RelA-regulated T6SS4 expression was collectively mediated by RovM and RovA. **(A)** Comparison of β-galactosidase activities of T6SS4 promoter of Δ*relA*Δ*rovA* double mutant and Δ*relA*Δ*rovA*Δ*rovM* triple mutant complemented with *relA* grown to stationary phase in M9 medium. **(B)** Comparison of β-galactosidase activities of T6SS4 promoter of Δ*rovA*, Δ*rovM* and Δ*rovA*Δ*rovM* mutants overexpressing *relA* grown to stationary phase in M9 medium. Data represent the mean ± SD of three biological replicates, each of which was performed with three technical replicates. ***: $p < 0.001$, **: $p < 0.01$, n.s.: not significant.

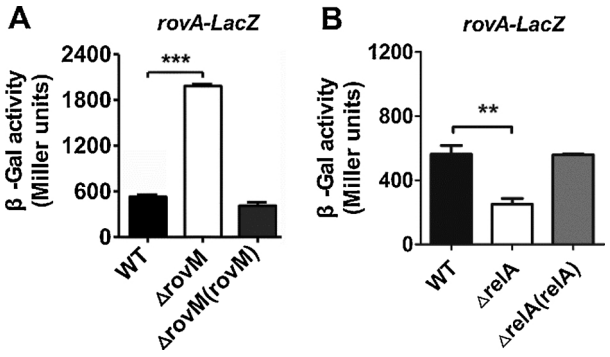

Fig. 7. (A) β-galactosidase analyses of *rovA* promoter activities in the *Y. pseudotuberculosis* wild-type, Δ*rovM* mutant and the complemented strain Δ*rovM* (*rovM*) grown to stationary phase in M9 medium. **(B)** β-galactosidase analyses of *rovA* promoter activities in the *Y. pseudotuberculosis* wild-type, Δ*relA* mutant and the complemented strain Δ*relA*(*relA*) grown to logarithmic phase in M9 medium. Data represent the mean ± SD of three biological replicates, each of which was performed with three technical replicates. ***: $p < 0.001$, **: $p < 0.01$.

Consistent with this report, the *rovA* promoter activity was significantly increased in Δ*rovM* compared to the WT (Fig. 7A). *rovA* expression was decreased in Δ*relA* according to the transcriptomics result (Δ*relA*/WT*100% = 51.63%, $\log_2$(Δ*relA*/WT) = -0.9537), and this was further confirmed by β-galactosidase assay. Deletion of *relA* markedly reduced *rovA* promoter activity, and which was restored in the Δ*relA*(*relA*) complemented strain (Fig. 7B).

4. Discussion

The stringent response (SR), which is controlled by the alarmone (p)ppGpp, is a global regulatory mechanism induced by nutrient starvation and multiple stresses (Gaca et al., 2013), and is crucial for bacterial virulence induction, differentiation, and persistence (Dahl et al., 2003; Nakanishi et al., 2006; Hesketh et al., 2007). In Gram-negative bacteria, synthesis of (p)ppGpp is catalyzed by the RelA/SpoT homologs, and RelA is the primary enzyme responsible for (p)ppGpp synthesis upon nutrient starvation (Potrykus and Cashel, 2008; Hauryliuk et al., 2015). Although SR was recognized as a central stress response system, only a few studies have explored the underlying protection mechanisms. Recently, Nguyen et al. reported that inactivation of the SR in *P. aeruginosa* causes increased endogenous ROS, and this excessive oxidative burden was due to impaired antioxidant defence and increased levels of pro-oxidant 4-hydroxyl-2-alkylquinolone (HAQ) molecules (Nguyen et al., 2011). The SR also regulates catalase expression and is required for hydrogen peroxide and antibiotic tolerance in *P. aeruginosa* (Khakimova et al., 2013). Moreover, it was shown that the SR regulatory protein DksA controls the production of reducing power to facilitate the repairment of biomolecules damaged by ROS under oxidative stress conditions (Henard et al., 2010).

Herein we found that T6SS4, a general stress response system in *Y. pseudotuberculosis*, was positively regulated by RelA, implicating it in the RelA mediated stress resistance (Fig. 1). Accumulating data indicate that T6SS is involved in resistant to multiple stress in various bacteria (Weber et al., 2009; Goldova et al., 2011; Records, 2011; Zhang et al., 2013). For example, a T6SS regulated by the general stress response regulator RpoS in *Vibrio anguillarum* was found to be involved in resistance to hydrogen peroxide, ethanol and low pH, though the underlying mechanism has not yet been revealed (Weber et al., 2009). Recently, we showed that the *Y. pseudotuberculosis* T6SS4 is required for the resistance to diverse stress conditions such as oxidative stress, acid stress, osmotic stress and heat stress, by importing Zn^{2+} from the environment to reduce intracellular ROS levels induced by these stresses (Wang et al., 2015). The T6SS4 in *Burkholderia thailandensis* has also been shown to be involved in the resistance to multiple stresses by reducing intracellular ROS levels via importing antioxidative ions of Mn^{2+} (Si et al., 2017a) and Zn^{2+} (Si et al., 2017b). These findings support the notion that SR contributes to bacterial resistance to multiple adverse stresses by increasing the expression of various antioxidant systems to mitigate the toxicity effects of ROS produced under these unfavourable conditions.

Consistent with the finding that the *Y. pseudotuberculosis* T6SS-4 functions to combat a broad range of adverse stresses, its expression is regulated by multiple regulators responding to various environmental stimuli, including the general stress response regulator RpoS (Weber et al., 2009; Guan et al., 2015), the osmotic/acid stress regulator OmpR (Gueguen et al., 2013; Zhang et al., 2013), the global oxidative stress regulator OxyR (Wang et al., 2015), and the zinc-responsive transcriptional regulator ZntR (Wang et al., 2017). These overlapping regulatory networks allow immediately adaptation of bacteria to ever-changing environments through activating the expression of T6SS4. Recently, RovM, a LysR-type global regulator sensing nutrition status, was found acts as both an activator and a repressor fine-tuning the expression of various acid survival systems such as T6SS4, AR3 and urease in *Y. pseudotuberculosis* in response to the availability of nutrients (Song et al., 2015; Dai et al., 2018). RovM also acts as a motile-

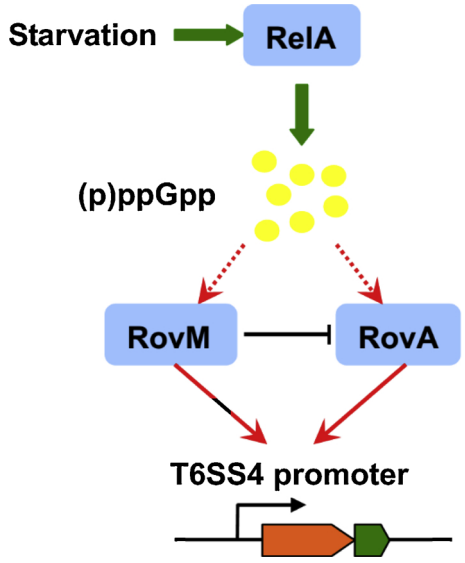

Fig. 8. Model of RelA regulation of T6SS4 mediated by RovM/RovA.

sessile state switch, regulates motility and biofilm formation based on nutrient availability (Zhao et al., 2017). Since T6SS4 is positively regulated by RovM, it is possible that the SR controls T6SS4 through this intermediary regulator involved in nutrition sensing. Notably, the SR controls RovM expression in *Y. pseudotuberculosis*. However, RovM is not the only regulator to mediate the regulation of T6SS4 by RelA, since RelA can still activate T6SS4 expression in the Δ*rovM* mutant, unless RovA, a RovM controlled regulator was further deleted in the Δ*rovM* mutant (Fig. 6). Thus, the (p)ppGpp synthase RelA regulates T6SS4 expression by the mediation of the RovM and RovA regulators. This is consistent with previous findings that (p)ppGpp synthases influences bacterial phenotypes by controlling the expression of many other regulators such as the cAMP receptor protein CRP, the flagellar master regulator FlhDC, and the integration host factor IHF (Aviv et al., 1994; Johansson et al., 2000; Lemke et al., 2009).

The MarR-type global regulator RovA of *Yersinia* has been shown to control the expression of multiple metabolic, stress and virulence genes that are required for environmental adaptation and pathogenesis (Nagel et al., 2001; Ellison et al., 2004; Cathelyn et al., 2006). Expression of *Yersinia* RovA is directly repressed by RovM, which itself is controlled by the Csr system in response to nutrition status (Heroven and Dersch, 2006). Previously it was reported that RovA indirectly regulates T6SS4 in *Y. pestis* CO92 (Cathelyn et al., 2006). However, in this study we found that RovA regulates T6SS4 expression in a direct manner in *Y. pseudotuberculosis*, and identified two RovA-binding sites with AT-rich sequences on the T6SS4 promoter (Fig. 5). Recently, RovA was recognized to be a proteinaceous thermometer that senses temperature shifts directly through alterations in protein conformation thereby modulating its DNA-binding capacity (Herbst et al., 2009). Since the expression of T6SS4 is also temperature dependent and significantly induced at 26 versus 37 °C (Zhang et al., 2011), it's interesting to investigate whether the temperature dependent T6SS4 expression was mediated by RovA in the future.

As a nutrient response regulator, the expression of RovM itself is nutrient-dependent and was shown to be controlled by the carbon storage regulator system CsrABC (Heroven et al., 2008). Under nutrient limited condition, CsrA activates RovM expression, leading to repression of RovA. The RNA-binding protein CsrA activates expression of the RovM protein, which in turn leads to a significant reduction in RovA levels. In contrast, the small regulatory RNAs CsrB and CsrC sequester CsrA and prevent it from activating RovM expression (Heroven et al., 2008). Both Csr system and SR system are the key global regulatory networks of bacteria for sensing nutrient availability (Edwards et al., 2011). Interestingly, in this study we observed that T6SS4 expression was not only regulated by the Csr system, but also regulated by the SR factor RelA. Thus, RovM acts as a central regulator to integrate the starvation signals sensed by the Csr system and SR system, and activate the general stress response system T6SS4 to adapt bacterial cells to adverse environments. Although RovA is dispensable for activation T6SS4 expression under nutrition limited conditions, it may play crucial roles in mediation of the temperature dependent expression of T6SS4 under nutrient rich conditions.

Although SR has been well-known to be a conserved regulatory mechanism that coordinates physiological adaptations to nutrient starvation and multiple stresses, the underlying mechanisms remain poorly understood. Based on our results, we propose a model whereby RelA positively regulates the expression of the general stress response T6SS4 system to combat various stresses in response to nutrition starvation, coordinately mediated by the RovM/RovA regulators (Fig. 8). These findings revealed not only the important role of T6SS4 in SR induced stress resistance, but also a new pathway to regulate T6SS4 expression in response to starvation stress.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2018.12.002.

References

Aubert, D.F., Xu, H., Yang, J., Shi, X., Gao, W., Li, L., Bisaro, F., Chen, S., Valvano, M.A., Shao, F., 2016. A *Burkholderia* type VI effector deamidates rho GTPases to activate the pyrin inflammasome and trigger inflammation. Cell Host Microbe 19 (5), 664–674.
Aviv, M., Giladi, H., Schreiber, G., Oppenheim, A.B., Glaser, G., 1994. Expression of the genes coding for the *Escherichia coli* integration host factor are controlled by growth phase, *rpoS*, ppGpp and by autoregulation. Mol. Microbiol. 14 (5), 1021–1031.
Cathelyn, J.S., Crosby, S.D., Lathem, W.W., Goldman, W.E., Miller, V.L., 2006. RovA, a global regulator of *Yersinia pestis*, specifically required for bubonic plague. Proc. Natl. Acad. Sci. U.S.A. 103 (36), 13514–13519.
Dahl, J.L., Kraus, C.N., Boshoff, H.I., Doan, B., Foley, K., Avarbock, D., Kaplan, G., Mizrahi, V., Rubin, H., Barry 3rd., C.E., 2003. The role of RelMtb-mediated adaptation to stationary phase in long-term persistence of *Mycobacterium tuberculosis* in mice. Proc. Natl. Acad. Sci. U.S.A. 100 (17), 10026–10031.
Dai, Q., Xu, L., Xiao, L., Zhu, K., Song, Y., Li, C., Zhu, L., Shen, X., Wang, Y., 2018. RovM and CsrA negatively regulate urease expression in *Yersinia pseudotuberculosis*. Front. Microbiol. 9, 348.
Dong, T.G., Ho, B.T., Yoder-Himes, D.R., Mekalanos, J.J., 2013. Identification of T6SS-dependent effector and immunity proteins by Tn-seq in *Vibrio cholerae*. Proc. Natl. Acad. Sci. U.S.A. 110 (7), 2623–2628.
Dong, T.G., Dong, S., Catalano, C., Moore, R., Liang, X., Mekalanos, J.J., 2015. Generation of reactive oxygen species by lethal attacks from competing microbes. Proc. Natl. Acad. Sci. U.S.A. 112 (7), 2181–2186.
Edwards, A.N., Patterson-Fortin, L.M., Vakulskas, C.A., Mercante, J.W., Potrykus, K., Vinella, D., Camacho, M.I., Fields, J.A., Thompson, S.A., Georgellis, D., Cashel, M., Babitzke, P., Romeo, T., 2011. Circuitry linking the Csr and stringent response global regulatory systems. Mol. Microbiol. 80 (6), 1561–1580.
Ellison, D.W., Lawrenz, M.B., Miller, V.L., 2004. Invasin and beyond: regulation of *Yersinia* virulence by RovA. Trends Microbiol. 12 (6), 296–300.
Gaca, A.O., Kajfasz, J.K., Miller, J.H., Liu, K., Wang, J.D., Abranches, J., Lemos, J.A., 2013. Basal levels of (p)ppGpp in *Enterococcus faecalis*: the magic beyond the stringent response. mBio 4 (5), e00646–13.
Goldova, J., Ulrych, A., Hercik, K., Branny, P., 2011. A eukaryotic-type signalling system of *Pseudomonas aeruginosa* contributes to oxidative stress resistance, intracellular survival and virulence. BMC Genomics 12, 437.
Guan, J., Xiao, X., Xu, S., Gao, F., Wang, J., Wang, T., Song, Y., Pan, J., Shen, X., Wang, Y., 2015. Roles of RpoS in *Yersinia pseudotuberculosis* stress survival, motility, biofilm formation and type VI secretion system expression. J. Microbiol. 53 (9), 633–642.
Gueguen, E., Durand, E., Zhang, X.Y., d'Amalric, Q., Journet, L., Cascales, E., 2013. Expression of a *Yersinia pseudotuberculosis* type VI secretion system is responsive to envelope stresses through the OmpR transcriptional activator. PLoS One 8 (6), e66615.
Hachani, A., Wood, T.E., Filloux, A., 2016. Type VI secretion and anti-host effectors. Curr. Opin. Microbiol. 29, 81–93.
Hauryliuk, V., Atkinson, G.C., Murakami, K.S., Tenson, T., Gerdes, K., 2015. Recent

functional insights into the role of (p)ppGpp in bacterial physiology. Nat. Rev. Microbiol. 13 (5), 298–309.
Henard, C.A., Bourret, T.J., Song, M., Vazquez-Torres, A., 2010. Control of redox balance by the stringent response regulatory protein promotes antioxidant defenses of Salmonella. J. Biol. Chem. 285 (47), 36785–36793.
Herbst, K., Bujara, M., Heroven, A.K., Opitz, W., Weichert, M., Zimmermann, A., Dersch, P., 2009. Intrinsic thermal sensing controls proteolysis of *Yersinia* virulence regulator RovA. PLoS Pathog. 5 (5), e1000435.
Heroven, A.K., Dersch, P., 2006. Rov M, a novel LysR-type regulator of the virulence activator gene *rovA*, controls cell invasion, virulence and motility of *Yersinia pseudotuberculosis*. Mol. Microbiol. 62 (5), 1469–1483.
Heroven, A.K., Nagel, G., Tran, H.J., Parr, S., Dersch, P., 2004. RovA is autoregulated and antagonizes H-NS-mediated silencing of invasin and *rovA* expression in *Yersinia pseudotuberculosis*. Mol. Microbiol. 53 (3), 871–888.
Heroven, A.K., Bohme, K., Rohde, M., Dersch, P., 2008. A Csr-type regulatory system, including small non-coding RNAs, regulates the global virulence regulator RovA of *Yersinia pseudotuberculosis* through RovM. Mol. Microbiol. 68 (5), 1179–1195.
Hesketh, A., Chen, W.J., Ryding, J., Chang, S., Bibb, M., 2007. The global role of ppGpp synthesis in morphological differentiation and antibiotic production in *Streptomyces coelicolor* A3(2). Genome Biol. 8 (8), R161.
Ho, B.T., Dong, T.G., Mekalanos, J.J., 2014. A view to a kill: the bacterial type VI secretion system. Cell Host Microbe 15 (1), 9–21.
Jiang, F., Wang, X., Wang, B., Chen, L., Zhao, Z., Waterfield, N.R., Yang, G., Jin, Q., 2016. The *Pseudomonas aeruginosa* type VI secretion PGAP1-like effector induces host autophagy by activating endoplasmic reticulum stress. Cell Rep. 16 (6), 1502–1509.
Johansson, J., Balsalobre, C., Wang, S.Y., Urbonaviciene, J., Jin, D.J., Sonden, B., Uhlin, B.E., 2000. Nucleoid proteins stimulate stringently controlled bacterial promoters: a link between the cAMP-CRP and the (p)ppGpp regulons in *Escherichia coli*. Cell 102 (4), 475–485.
Khakimova, M., Ahlgren, H.G., Harrison, J.J., English, A.M., Nguyen, D., 2013. The stringent response controls catalases in *Pseudomonas aeruginosa* and is required for hydrogen peroxide and antibiotic tolerance. J. Bacteriol. 195 (9), 2011–2020.
Lemke, J.J., Durfee, T., Gourse, R.L., 2009. DksA and ppGpp directly regulate transcription of the *Escherichia coli* flagellar cascade. Mol. Microbiol. 74 (6), 1368–1379.
Lin, J., Zhang, W., Cheng, J., Yang, X., Zhu, K., Wang, Y., Wei, G., Qian, P.Y., Luo, Z.Q., Shen, X., 2017. A *Pseudomonas* T6SS effector recruits PQS-containing outer membrane vesicles for iron acquisition. Nat. Commun. 8, 14888.
Ma, L.S., Hachani, A., Lin, J.S., Filloux, A., Lai, E.M., 2014. *Agrobacterium tumefaciens* deploys a superfamily of type VI secretion DNase effectors as weapons for interbacterial competition in planta. Cell Host Microbe 16 (1), 94–104.
Miller, J.H., 1992. A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
Nagel, G., Lahrz, A., Dersch, P., 2001. Environmental control of invasin expression in *Yersinia pseudotuberculosis* is mediated by regulation of RovA, a transcriptional activator of the SlyA/Hor family. Mol. Microbiol. 41 (6), 1249–1269.
Nakanishi, N., Abe, H., Ogura, Y., Hayashi, T., Tashiro, K., Kuhara, S., Sugimoto, N., 2006. Tobe T. ppGpp with DksA controls gene expression in the locus of enterocyte effacement (LEE) pathogenicity island of enterohaemorrhagic *Escherichia coli* through activation of two virulence regulatory genes. Mol. Microbiol. 61 (1), 194–205.
Nguyen, D., Joshi-Datar, A., Lepine, F., Bauerle, E., Olakanmi, O., Beer, K., McKay, G., Siehnel, R., Schafhauser, J., Wang, Y., Britigan, B.E., Singh, P.K., 2011. Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria. Science 334 (6058), 982–986.
Okada, Y., Makino, S., Tobe, T., Okada, N., Yamazaki, S., 2002. Cloning of rel from *Listeria monocytogenes* as an osmotolerance involvement gene. Appl. Environ. Microbiol. 68 (4), 1541–1547.
Potrykus, K., Cashel, M., 2008. (p)ppGpp: still magical? Annu. Rev. Microbiol. 62, 35–51.
Records, A.R., 2011. The type VI secretion system: a multipurpose delivery system with a phage-like machinery. Mol. Plant-microbe Interact. 24 (7), 751–757.
Russell, A.B., Hood, R.D., Bui, N.K., LeRoux, M., Vollmer, W., Mougous, J.D., 2011. Type VI secretion delivers bacteriolytic effectors to target cells. Nature 475 (7356), 343–347.
Russell, A.B., LeRoux, M., Hathazi, K., Agnello, D.M., Ishikawa, T., Wiggins, P.A., Wai, S.N., Mougous, J.D., 2013. Diverse type VI secretion phospholipases are functionally plastic antibacterial effectors. Nature 496 (7446), 508–512.
Russell, A.B., Peterson, S.B., Mougous, J.D., 2014. Type VI secretion system effectors: poisons with a purpose. Nat. Rev. Microbiol. 12 (2), 137–148.
Si, M.R., Zhang, L., Yang, Z.F., Xu, Y.X., Liu, Y.B., Jiang, C.Y., Wang, Y., Shen, X.H., Liu, S.J., 2014. NrdH Redoxin enhances resistance to multiple oxidative stresses by acting as a peroxidase cofactor in *Corynebacterium glutamicum*. Appl. Environ. Microbiol. 80 (5), 1750–1762.
Si, M., Zhang, L., Chaudhry, M.T., Ding, W., Xu, Y., Chen, C., Akbar, A., Shen, X., Liu, S.J., 2015. *Corynebacterium glutamicum* methionine sulfoxide reductase A uses both mycoredoxin and thioredoxin for regeneration and oxidative stress resistance. Appl. Environ. Microbiol. 81 (8), 2781–2796.
Si, M., Zhao, C., Burkinshaw, B., Zhang, B., Wei, D., Wang, Y., Dong, T.G., Shen, X., 2017a. Manganese scavenging and oxidative stress response mediated by type VI secretion system in *Burkholderia thailandensis*. Proc. Natl. Acad. Sci. U.S.A. 114 (11), E2233–E2242.
Si, M., Wang, Y., Zhang, B., Zhao, C., Kang, Y., Bai, H., Wei, D., Zhu, L., Zhang, L., Dong, T.G., Shen, X., 2017b. The type VI secretion system engages a redox-regulated dual-functional heme transporter for zinc acquisition. Cell Rep. 20 (4), 949–959.
Song, Y., Xiao, X., Li, C., Wang, T., Zhao, R., Zhang, W., Zhang, L., Wang, Y., Shen, X., 2015. The dual transcriptional regulator RovM regulates the expression of AR3- and T6SS4-dependent acid survival systems in response to nutritional status in *Yersinia pseudotuberculosis*. Environ. Microbiol. 17 (11), 4631–4645.
Traxler, M.F., Summers, S.M., Nguyen, H.T., Zacharia, V.M., Hightower, G.A., Smith, J.T., Conway, T., 2008. The global, ppGpp-mediated stringent response to amino acid starvation in *Escherichia coli*. Mol. Microbiol. 68 (5), 1128–1148.
Vercruysse, M., Fauvart, M., Jans, A., Beullens, S., Braeken, K., Cloots, L., Engelen, K., Marchal, K., Michiels, J., 2011. Stress response regulators identified through genome-wide transcriptome analysis of the (p)ppGpp-dependent response in *Rhizobium etli*. Genome Biol. 12 (2), R17.
Wang, Y., Cen, X.F., Zhao, G.P., Wang, J., 2012. Characterization of a new GlnR binding box in the promoter of *amtB* in *Streptomyces coelicolor* inferred a PhoP/GlnR competitive binding mechanism for transcriptional regulation of *amtB*. J. Bacteriol. 194 (19), 5237–5244.
Wang, T., Si, M., Song, Y., Zhu, W., Gao, F., Wang, Y., Zhang, L., Zhang, W., Wei, G., Luo, Z.Q., Shen, X., 2015. Type VI secretion system transports Zn^{2+} to combat multiple stresses and host immunity. PLoS Pathog. 11 (7), e1005020.
Wang, T., Chen, K., Gao, F., Kang, Y., Chaudhry, M.T., Wang, Z., Wang, Y., Shen, X., 2017. ZntR positively regulates T6SS4 expression in *Yersinia pseudotuberculosis*. J. Microbiol. 55 (6), 448–456.
Weber, B., Hasic, M., Chen, C., Wai, S.N., Milton, D.L., 2009. Type VI secretion modulates quorum sensing and stress response in *Vibrio anguillarum*. Environ. Microbiol. 11 (12), 3018–3028.
Xu, S., Peng, Z., Cui, B., Wang, T., Song, Y., Zhang, L., Wei, G., Wang, Y., Shen, X., 2014. FliS modulates FlgM activity by acting as a non-canonical chaperone to control late flagellar gene expression, motility and biofilm formation in *Yersinia pseudotuberculosis*. Environ. Microbiol. 16 (4), 1090–1104.
Yan, X., Zhao, C., Budin-Verneuil, A., Hartke, A., Rince, A., Gilmore, M.S., Auffray, Y., Pichereau, V., 2009. The (p)ppGpp synthetase RelA contributes to stress adaptation and virulence in *Enterococcus faecalis* V583. Microbiology 155 (Pt 10), 3226–3237.
Yang, X., Pan, J., Wang, Y., Shen, X., 2018. Type VI secretion systems present new insights on pathogenic *Yersinia*. Front. Cell. Infect. Microbiol. 8 (260).
Zhang, W., Xu, S., Li, J., Shen, X., Wang, Y., Yuan, Z., 2011. Modulation of a thermoregulated type VI secretion system by AHL-dependent quorum sensing in *Yersinia pseudotuberculosis*. Arch. Microbiol. 193 (5), 351–363.
Zhang, W., Wang, Y., Song, Y., Wang, T., Xu, S., Peng, Z., Lin, X., Zhang, L., Shen, X., 2013. A type VI secretion system regulated by OmpR in *Yersinia pseudotuberculosis* functions to maintain intracellular pH homeostasis. Environ. Microbiol. 15 (2), 557–569.
Zhao, R., Song, Y., Dai, Q., Kang, Y., Pan, J., Zhu, L., Zhang, L., Wang, Y., Shen, X., 2017. A starvation-induced regulator, RovM, acts as a switch for planktonic/biofilm state transition in *Yersinia pseudotuberculosis*. Sci. Rep. 7 (1), 639.