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Roles of RpoS in *Yersinia pseudotuberculosis* stress survival, motility, biofilm formation and type VI secretion system expression[§]

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RpoS (σ^S), the stationary phase/stress σ factor, controls the expression of a large number of genes involved in cellular responses to a variety of stresses. However, the role of RpoS appears to differ in different bacteria. While RpoS is an important regulator of flagellum biosynthesis, it is associated with biofilm development in *Edwardsiella tarda*. Biofilms are dense communities formed by bacteria and are important for microbe survival under unfavorable conditions. The type VI secretion system (T6SS) discovered recently is reportedly associated with several phenotypes, ranging from biofilm formation to stress sensing. For example, *Vibrio anguillarum* T6SS was proposed to serve as a sensor for extracytoplasmic signals and modulates RpoS expression and stress response. In this study, we investigated the physiological roles of RpoS in *Yersinia pseudotuberculosis*, including bacterial survival under stress conditions, flagella formation, biofilm development and T6SS expression. We found that RpoS is important in resistance to multiple stressors–including H_2O_2, acid, osmotic and heat shock–in *Y. pseudotuberculosis*. In addition, our study showed that RpoS not only modulates the expression of T6SS but also regulates flagellum formation by positively controlling the flagellar master regulatory gene *flhDC*, and affects the formation of biofilm on *Caenorhabditis elegans* by regulating the synthesis of exopolysaccharides. Taken together, these results show that RpoS plays a central role in cell fitness under several adverse conditions in *Y. pseudotuberculosis*.

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Introduction

RpoS (σ^S), the product of the *rpoS* gene, is an alternate σ factor considered the master regulator of gene expression in the stationary phase (Lacour and Landini, 2004). RpoS, together with the RNA polymerase core enzyme, forms the RNA polymerase holoenzyme, which initiates the transcription process. The well-studied alternative σ factor RpoS is also widely considered to be the general stress response σ factor. RpoS directs RNA polymerase binding to the promoters of *rpoS*-dependent genes. Most of these genes are necessary for the transition to stationary phase (Hengge-Aronis, 2002) or for resistance to various stress conditions, including starvation, the presence of reactive oxygen species, heat and osmotic stresses (Landini *et al.*, 2014).

In addition to its role in the stress response, RpoS is required for flagellum formation, virulence and quorum-sensing (QS) regulation in some bacteria (Tian *et al.*, 2008; Sommerfeldt *et al.*, 2009; Yan *et al.*, 2009). In *Escherichia coli*, RpoS regulates more than 100 genes involved in survival and virulence and in *Vibrio alginolyticus*, RpoS positively regulates LuxS QS by modulating the AI-2 (an autoinducer secreted by the QS system of Gram-negative bacteria) biosynthesis system. The flagellum, the locomotion organ of bacteria, is crucial for their moving to favorable environments and avoiding harmful substances. When facing unfavorable conditions, bacteria can also protect themselves by forming biofilms. Biofilm formation is a social behavior that produces favorable conditions for bacterial survival in the natural environment and represents a mode of protection that allows cells to survive in various disadvantageous environments (Donlan and Costerton, 2002). Numerous Gram-negative bacteria depend on flagellum-mediated motility for specific stages of biofilm formation. For example, in *Campylobacter jejuni*, the flagellar filament is kinetically aided by motility and required for attachment during biofilm formation (Svensson *et al.*, 2014). Additionally, QS regulates biofilm formation in *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, and *Burkholderia cenocepacia* by regulating the production of extracellular DNA and lectins, which function as biofilm matrix components (Fazli *et al.*, 2014; Packiavathy *et al.*, 2014; Perez-Montano *et al.*, 2014). However, in *Y. pseudotuberculosis*, whether RpoS affects flagellum synthesis and biofilm formation remains unknown.

Bacterial pathogens employ a variety of secretion systems to overcome the host immune system and improve their

chances of survival. The recently identified type VI secretion system (T6SS) is widely distributed in Gram-negative bacteria and is structurally and functionally similar to contractile phage tail sheath (Records, 2011). One striking feature of the T6SS is that many genomes harbor multiple T6SS gene clusters that are thought to be involved in various functions. T6SSs reportedly play versatile physiological roles in areas such as host/symbiont communication, biofilm formation, interbacterial interactions and acute and chronic infections (Pukatzki *et al.*, 2009; Hood *et al.*, 2010). Interestingly, in recent studies, T6SS was suggested as essential for bacterial survival under stress conditions. The T6SS in *Vibrio anguillarum* is important for bacterial survival after exposure to ethanol and low pH treatment (Weber *et al.*, 2009). Additionally, in the *Vibrio cholerae* O1 strain A1552, the T6SS was activated when bacteria were grown under high osmolarity or low temperature (23°C) conditions, suggesting that the system may be important for the survival of *V. cholerae* in the environment (Ishikawa *et al.*, 2012). Four T6SS gene clusters believed to function differently under specific conditions have been identified in the enteric pathogen *Y. pseudotuberculosis*. Among the four T6SS clusters, T6SS4 was reported to be essential for bacterial survival under acidic and osmotic stresses and for resistance to deoxycholate (Gueguen *et al.*, 2013; Zhang *et al.*, 2013).

Although RpoS has been shown to be important in the stress response and biofilm development, knowledge regarding its role in *Y. pseudotuberculosis* is limited. In this study we showed that RpoS, as an alternate σ factor, could facilitate survival of *Y. pseudotuberculosis* under H_2O_2, acid, osmotic and heat stress conditions. Besides the nine well-known σ^S-dependent stress-related genes, RpoS can also directly activate the expression of the stress-resistant T6SS4 in *Y. pseudotuberculosis*. The roles of RpoS in osmotic and acid resistance were mediated in part by T6SS4. Furthermore, the expression of $FlhD_4C_2$, the product of the flagellar master regulatory gene *flhDC*, was positively but indirectly controlled by RpoS. Biofilm production by *Yersinia* spp. is dependent on the *hmsHFRS* operon, which encodes proteins responsible for biosynthesis and translocation of biofilm exopolysaccharides through the cell envelope (Drace and Darby, 2008). In this study, we also found that RpoS affects biofilm formation by indirectly regulating *hmsHFRS* expression. Enteropathogenic *Yersinia* species must adapt and respond to a variety of environmental conditions and host-mediated assaults to establish a successful infection, which can be accomplished through transcriptional regulation of the genes required for particular stages of the adaptation and infection processes. Therefore, determining the function of the σ^S factor is necessary for controlling the diseases caused by *Y. pseudotuberculosis*.

Materials and Methods

Bacterial strains and growth conditions

Strains and plasmids used in this study are listed in Supplementary data Table S1. The *Y. pseudotuberculosis* strain YPIII was the parent of all derivatives used in this study. *Y. pseudotuberculosis* strains were cultivated in YLB medium (1% tryptone, 0.5% NaCl, and 0.5% yeast extract) with appropriate antibiotics when necessary. For all experiments in this study, *Y. pseudotuberculosis* strains were grown to stationary phase at 26°C unless stated. *E. coli* strains were cultivated in LB medium (Luria-Bertani) under 37°C with appropriate antibiotics when necessary. To construct the Δ*rpoS* deletion mutant, the wild-type YPIII was mated with *E. coli* S17-1λpir carrying pDM4-Δ*rpoS* and chromosomal integration was selected by plating on YLB agar plates supplemented with chloramphenicol. The Δ*rpoS* mutant was subsequently screened on YLB agar plates with 20% sucrose and confirmed by PCR and DNA sequencing as previously described (Zhang *et al.*, 2013). The concentration of antibiotics used in this study were: ampicillin 100 μg/ml, nalidixic acid 20 μg/ml, chloramphenicol 20 μg/ml, kanamycin 50 μg/ml.

Plasmid construction

Primers used in this study are listed in Supplementary data Table S2. To express His_6-tagged *rpoS* (*ypk_3425*), primers *rpoS*-F-*Bam*HI and *rpoS*-R-*Hin*dIII were used to amplify the *rpoS* gene fragment from genomic DNA of YPIII. The PCR product was digested with *Bam*HI/*Hin*dIII and inserted into the *Bam*HI/*Hin*dIII sites of pET28a resulting in plasmid pET-28a-*rpoS*. To express His_6-tagged RovM (Heroven and Dersch, 2006), primers *rovM*-F-*Nde*I and *rovM*-R-*Xho*I were used to amplify the *rovM* gene fragment from the YPIII genome. The PCR product was digested with *Nde*I/*Xho*I and inserted into the *Nde*I/*Xho*I sites of pET15b to generate pET15b-*rovM*. The *lacZ* fusion reporter vector pDM4-*T6SS4p*::*lacZ* and pDM4-*flhDCp*::*lacZ* were made in previous studies (Ding *et al.*, 2009; Zhang *et al.*, 2013). To construct the *lacZ* fusion reporter vector pDM4-*hmsHFRSp*::*lacZ*, primers *hmsHFRSp*-F-*Sal*I/*hmsHFRSp*-R-*Xba*I were used to amplify the 668 bp *hmsHFRS* (*ypk_2238-2241*) promoter fragment from YPIII genomic DNA. The PCR product was digested with *Sal*I/*Xba*I and inserted into similarly digested pDM4-*lacZ* to produce pDM4-*hmsHFRSp*::*lacZ*. To construct the T6SS4 promoter with RpoS-binding site mutation, overlap PCR was performed to replace the 8-bp consensus binding site (CCCCCTCC) with TTTTTCTT. Briefly, the primer pairs T6SS4p-F-*Sal*I/T6SS4p-BD-M1R and T6SS4p-BD-M2F/T6SS4p-R-*Xba*I were used to amplify the up-fragment and down-fragment of the T6SS4 promoter, respectively. Overlap PCR was carried out using the up-fragment and down-fragment as template and T6SS4p-F-*Sal*I/T6SS4p-R-*Xba*I as primer pair to obtain the DNA fragment T6SS4pM. This fragment was further digested with *Sal*I and *Xba*I and inserted into the similarly digested pDM4-*lacZ* to construct pDM4-*T6SS4pM*::*lacZ*. The plasmid pDM4-Δ*rpoS* was used to construct the *Y. pseudotuberculosis* Δ*rpoS* deletion mutant. The 736-bp upstream PCR product and 711-bp downstream PCR product of *rpoS* were amplified using primer pairs *rpoS*-up-F-*Sal*I/*rpoS*-up-R and *rpoS*-down-F/*rpoS*-down-R-*Bgl*II, respectively. The upstream and downstream PCR fragments were ligated by overlap PCR. The resulting PCR products were digested with *Sal*I and *Bgl*II, and inserted into the *Sal*I/*Bgl*II site of pDM4 to produce pDM4-Δ*rpoS*. To complement the Δ*rpoS* mutant, primers *rpoS*-F-*Pst*I/*rpoS*-R-*Bam*HI were used to amplify the *rpoS* gene fragment from

YPIII genomic DNA. The PCR product was digested with *Pst*I/*Bam*HI and was inserted into similarly digested pKT100 to produce pKT100-*rpoS*. To overexpress *flhDC* in the Δ*rpoS* mutant, plasmid pKT100-*flhDC* was constructed. Briefly, primers *flhDC*-F-*Sal*I/*flhDC*-R-*Bam*HI were used to amplify the *flhDC* gene fragment from YPIII genome. The PCR product of *flhDC* was digested with *Bam*HI/*Sal*I and was inserted into the *Bam*HI/*Sal*I sites of pKT100 to produce pKT100-*flhDC*. The integrity of the insert in all constructs was confirmed by DNA sequencing.

Overexpression and purification of recombinant protein

Recombinant plasmid pET28a-*rpoS* and pET15b-*rovM* were transformed into *E. coli* BL21(DE3) and *Trans*B(DE3) cells, respectively, and the recombinant strains were grown at 37°C to an OD_{600}~0.5. Then 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce expression. After incubating at 26°C for another 16 h, cells were harvested and resuspended with PBS buffer, broken by sonication until the suspension was translucent. Clear cell lysate was purified with Ni-NTA His×Bind Resin (Novagen) according to manufacturer's instructions. Purified recombinant proteins were dialyzed in phosphate-buffered saline (PBS) overnight at 4°C and then stored at -80°C until used. The concentration of protein was determined by using the Bradford Protein Assay Kit (Bio-Rad) according to the manufacturer's instructions, with bovine serum albumin (BSA) as standard.

RNA isolation and quantitative Real-Time PCR (qRT-PCR) analysis

All strains were grown to stationary-phase and then 1 ml cultures were collected by centrifugation. Total RNA was extracted from bacteria using EasyPure RNA Kit (TransGen Biotech) and treated with RNase-free DNase according to the manufacturer's protocol. The purity and concentration of the RNA was determined by gel electrophoresis and spectrophotometer (NanoDrop, Thermo Scientific). First-strand cDNA was reverse transcribed from 1 μg of total RNA with the TransScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech) with a little improvement: before reverse transcription happened, mixture were incubated at 65°C for 10 min and on ice for 5 min. Quantitative real-time PCR (qRT-PCR) was performed in CFX96 Real-Time PCR Detection System (Bio-Rad) with TransStart Top Green qPCR SuperMix (TransGen Biotech) following the manufacturer's specified protocol. Before qRT-PCR was conducted, all cDNAs were 250× diluted. For all primer sets (Supplementary data Table S2), 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. For standardization of results, the relative abundance of 16S rRNA was used as the internal standard. These assays were performed in triplicate at least three times, and error bars represent standard deviations.

Survival assays

Y. pseudotuberculosis strains were grown in YLB medium at 26°C to stationary phase. For acid stress assay, stains were diluted 50-fold into M9 medium (KH_2PO_4, 3 g/L; Na_2HPO_4, 6 g/L; NH_4Cl, 1 g/L; NaCl, 0.5 g/L; $CaCl_2$, 0.1 mM; $MgSO_4$, 1 mM; glucose, 0.2%) which were adjusted to pH 4.0 or pH 7.0, respectively. For H_2O_2 stress assay, after 50-fold diluted into M9 medium, H_2O_2 was added into the culture with the concentration of 1.5 mM. And for osmotic stress assay, strains were diluted 50-fold into M9 medium or the one containing 0.5 M NaCl, respectively. After incubating at 26°C for 1 h, cultures were serially diluted and plated onto YLB agar plates, colonies were counted after 24 h cultivation. For the heat shock assay, 50-fold diluted cultures were incubated at 42°C or 26°C for 30 min, serially diluted and then plated onto YLB agar plates. Percentage survival was calculated as follows: (CFU/ml of stressed cells/CFU/ml of untreated cells) ×100. Each experiment was done three times independently.

Construction of chromosomal fusion reporter strains and β -galactosidase assay

The *lacZ* fusion reporter vectors pDM4-*flhDCp*::*lacZ* (Ding *et al*., 2009), pDM4-*T6SS4p*::*lacZ* (Zhang *et al*., 2013), pDM4-*T6SS4pM*::*lacZ*, and pDM4-*hmsHFRSp*::*lacZ* were transformed into *E. coli* S17-1λ *pir* and then mated with *Y. pseudotuberculosis* strains. The chromosomal fusion strains were selected as described (Atkinson *et al*., 1999; Ding *et al*., 2009). The *lacZ* fusion reporter strains were grown to stationary phase at 26°C in YLB with appropriate antibiotics and β-galactosidase activity was determined using ONPG as substrate as described (Miller, 1992). The β-galactosidase data shown represent the mean of one representative assay done in triplicate, and error bars represent standard deviation. Statistical analysis was carried out with Student's *t*-test.

Electrophoretic mobility shift assay (EMSA)

EMSA was conducted as described (Lee and Gralla, 2001): 80 nM RpoS and 20 nM RNA polymerase core enzyme (New England Biolabs) were incubated on ice for 20 min, and then added to a 20 μl reaction mixture which contains 1 mM $MgSO_4$ 1 μl, 1 mM KCl 1 μl, 1% NP-40 1 μl, 10× binding buffer 2 μl, 50% glycerol 1 μl, target DNA 120 ng. Mixture was then incubated at room temperature for 30 min. 6% native gel was used to detect the mobility. As negative controls, only 80 nM RpoS or 20 nM RNA polymerase core enzyme was added to the reaction mixture, and a 700 bp DNA fragment amplified from the *rpoS* gene coding region with primers *rpoS*-F697 and *rpoS*-R697 was used as another control.

Motility assay and the transmission electron microscope observation of flagella

Semi-solid agar medium (1% tryptone, 0.5% NaCl, 0.3% Difco Bacto agar) was used in swimming motility assay. 1 μl bacterium solution was injected into semi-solid agar medium and incubated for 2 or 3 days under 22°C before observation (Atkinson *et al*., 1999). For the transmission electron microscopy, bacteria were grown on semi-solid agar slant for 7–8 h at 22°C and then at 28°C for 19 h. Then bacteria were washed off from the medium gently and stained with 2% sodium phosphotungstate before microscope observation (Xu *et al*., 2014a).

Biofilm assays on *Caenorhabditis elegans*

C. elegans were grown on NGM plates seeded with *E. coli* OP50. *Y. pseudotuberculosis* wild type, Δ*rpoS* mutant and the complementary strain labeled with the pKEN-GFP mutant3* plasmid were used in this part. 1 ml of stationary phase cultures were added on NGM plates respectively and grown under 30°C overnight. For the biofilm severity assay, young adult *C. elegans* were spreaded on the seeded plates and after 24 h incubation under 22°C, worms were washed off from the plates and examined under fluorescence microscope. Biofilm accumulation was divided into 4 levels, from level 0 to level 3. Level 0 represents no biofilm formation on *C. elegans*; level 1 indicates a little amount of biofilm formation around the worms' anterior; and level 2 by larger accumulation of biofilm around the anterior end and some may spread back from the head; level 3 denotes biofilm extend to other parts of the body besides the head (Atkinson *et al.*, 2011).

EPS quantification

Extracellular polysaccharide (EPS) was quantified as described by Doherty (1988). Cultures were grown to stationary phase in YLB medium and then 1 ml of the cultures was washed with M9, resuspended in 100 ml M9 medium. After incubating at 30°C for 24 to 72 h, cells were pelleted. 0.3 volume of 1% CTAB was added at room temperature to precipitate the polysaccharides and then redissolved in 10% NaCl. Samples were diluted at least 10 fold before quantified by phenol-vitriol method. Briefly, glucose dried to constant weight was used as the standard sample. Glucose solutions with different concentration were mixed with 1 ml 6% phenol solution and 5 ml vitriol, and then measured by recording the absorbance at 490 nm using a spectrophotometer. As for samples, 2 ml 6 mol/L HCl was added to each sample, incubated at 96°C for 2 h and then adding 2 ml 6 mol/L NaOH. Different volumes of the mixture were then supplemented to 2 ml with ddH_2O and later 1 ml 6% phenol, 5 ml vitriol were added. After incubating at room temperature for 20 min, polysaccharides were quantified at A_{490}. Total cell proteins were acquired using ultrasonic cell disruption system and quantified. All assays were done in triplicate independently.

Results

RpoS is involved in resistance to multiple stresses in *Y. pseudotuberculosis*

RpoS is also considered the general stress response σ factor. Since RpoS is crucial for survival of *E. coli* in the stationary phase by promoting resistance to multiple stressors (Landini *et al.*, 2014), we investigated whether it has the same effect in *Y. pseudotuberculosis* by constructing an *rpoS* deletion mutant. As shown in Supplementary data Fig. S1, deletion of the *rpoS* gene did not affect the growth rate of *Y. pseudotuberculosis* under normal conditions. However, the survival rates of the Δ*rpoS* mutant under 1.5 mM H_2O_2, pH 4.0, 0.5 M NaCl and 42°C heat shock decreased compared with the wild-type strain, and the defect was highly recovered in the complementary strain (Fig. 1). This indicates that RpoS protects *Y. pseudotuberculosis* cells from a variety of stressors including H_2O_2, acid, osmotic and heat shock.

Identification of σ^S-dependent stress-related genes

As mentioned above, RpoS promotes cell resistance to multiple stressors in *Y. pseudotuberculosis*. Thus, using quantitative real-time PCR (qRT-PCR) analysis we investigated whether RpoS regulates several well-known stress-related genes. The expression levels of nine stress-related genes during stationary phase in the *Y. pseudotuberculosis* wild-type strain and the Δ*rpoS* mutant were determined. To our knowledge, universal stress protein B (*uspB*) responses to different starvation and stress conditions (Farewell *et al.*, 1998); bacterioferritin (*bfr*) and DNA-binding proteins from starved cells (*dps*) protect against ROS-induced oxidative damage (Zeth, 2012); one of the fumarases, *fumC*, enhances cell survival under highly oxidative conditions (Park and Gunsalus, 1995); *katE* is a second chromosomally encoded catalase induced by weak acids (Schellhorn and Stones, 1992); *gadA* encodes glutamate decarboxylase, which is required by the acid resistance (AR) system 2 and AR2 protects the bacteria from acidic conditions (pH 2.5 to 4.5) (Castanie-Cornet *et al.*, 1999); *gadW* and *gadX* are AraC-family regulators and protect cells from acid stress (Sayed *et al.*, 2007). As shown

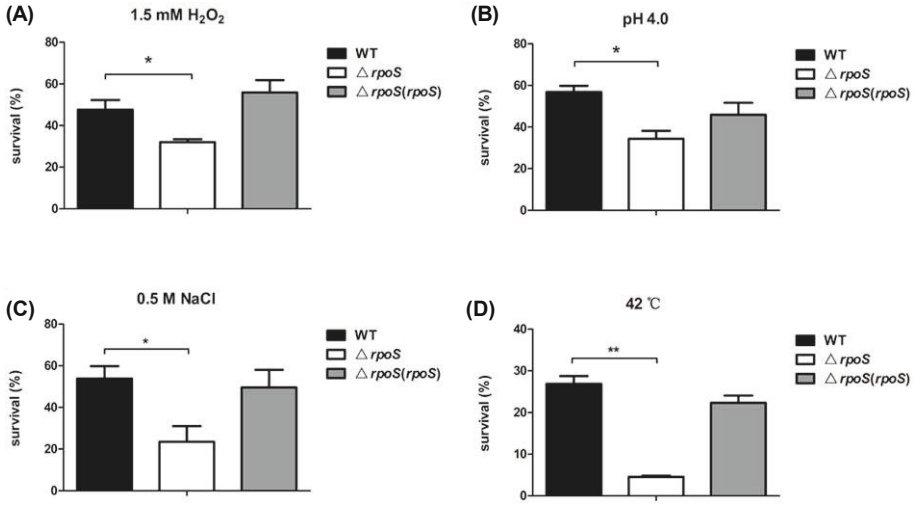

Fig. 1. Effects of *rpoS* deletion on stress resistance in *Y. pseudotuberculosis*. Survival rates of the *Y. pseudotuberculosis* wild-type (WT), Δ*rpoS* mutant and the complementary strain after exposure to 1.5 mM H_2O_2 (A), pH 4.0 (B), 0.5 M NaCl (C), and heat treatment (D). The asterisks indicate significant differences between the WT and Δ*rpoS* strains, (*t*-test and nonparametric-test, * $P \leq 0.05$). All experiments were performed independently in triplicate. Error bars are standard deviations of the mean of three replicates.

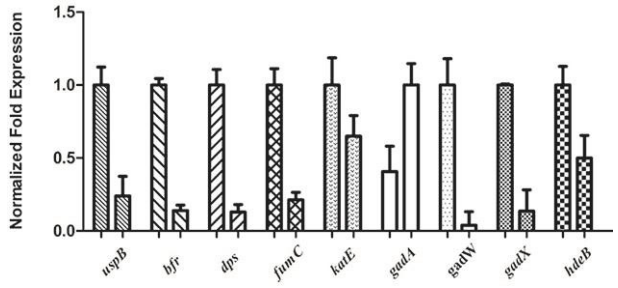

Fig. 2. Identification of σ^S-dependent stress-related genes in *Y. pseudotuberculosis*. Expression of the universal stress protein B gene (*uspB*), acid resistance genes (*gadA*, *gadW*, *gadX*, and *hdeB*) and oxidative stress resistance genes (*fumC*, *dps*, *katE*, and *bfr*) in the *Y. pseudotuberculosis* wild-type and Δ*rpoS* mutant were analyzed using qRT-PCR. For every gene shown, the first bar represents the wild-type and the second bar indicates the mutant. Error bars are standard deviations of the mean of three replicates.

in Fig. 2, the expression levels of these nine genes were regulated by RpoS. Interestingly, with the exception of *gadA*, the other eight genes were positively regulated by RpoS, indicating that RpoS has an important effect on the regulation of stress response genes in *Y. pseudotuberculosis*, and thus allows the cells to survive in unfavorable environments.

RpoS positively regulates T6SS4 expression

Since T6SS4 is involved in resistance to multiple stresses (Gueguen *et al*., 2013; Zhang *et al*., 2013), we hypothesized that T6SS4 may function as a general stress response system and is controlled by RpoS to facilitate survival of *Y. pseudotuberculosis* cells under stress conditions. To investigate the role of RpoS in T6SS4 expression, we introduced a single copy of the transcriptional fusion *T6SS4p::lacZ* into the chromosomes of *Y. pseudotuberculosis* wild-type, Δ*rpoS* mutant and the complementary strain Δ*rpoS*(*rpoS*). The activity of LacZ was then quantified as described (Miller, 1992). As shown in Fig. 3A, the β-galactosidase activity in the Δ*rpoS* mutant decreased by 39.8% compared with the wild-type; moreover, the decrease was almost completely recovered by cloning of a plasmid expressing RpoS (pKT100-*rpoS*) into the Δ*rpoS* mutant. These data demonstrate that RpoS positively regulates T6SS4 expression.

We further investigated whether RpoS regulates T6SS4 expression by directly binding to the T6SS4 promoter. The interaction between RpoS and the T6SS4 promoter was studied using an electrophoretic mobility shift assay (EMSA). Incubation of *E. coli* RNA polymerase core enzyme, His_6-RpoS and the T6SS4 promoter together led to retarded mobility. In contrast, incubating only the RNA polymerase core enzyme or His_6-RpoS with the T6SS4 promoter did not show detectable binding. As the negative control, a 700 bp (the length of the T6SS4 promoter) PCR fragment amplified from the *rpoS*-coding region was used; no retarded mobility was observed, although both the RNA polymerase core enzyme and His_6-RpoS were added (Fig. 3B). Consistent with this result, an RpoS binding site was identified in the T6SS4 promoter (Supplementary data Fig. S2). Collectively, these results indicate that RpoS positively regulates T6SS4 expression by directly binding to the T6SS4 promoter. This conclusion was further confirmed by the finding that mutation of the RpoS binding site in the T6SS4 promoter completely abolished RpoS-dependent activation of the T6SS4 promoter in the β-galactosidase assay (Fig. 3A). However, compared with the two reported regulation systems that activate T6SS4 expression, the YpsRI QS system (Atkinson *et al*., 2008) and the OmpR regulator (Zhang *et al*., 2013), RpoS plays a lesser

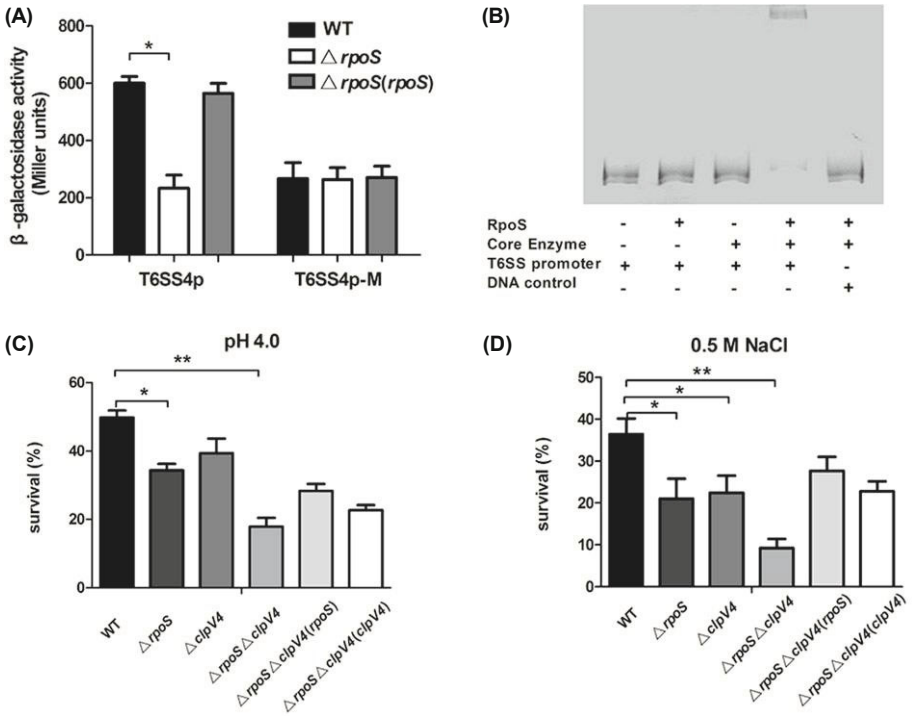

Fig. 3. RpoS regulates T6SS4 in *Y. pseudotuberculosis*. (A) Promoter activities were analyzed for the T6SS4 promoter (T6SS4p) and the T6SS4 promoter with mutation in RpoS binding site (T6SS4p-M) in the wild type, Δ*rpoS* mutant, and the complemented strain Δ*rpoS*(*rpoS*). Mean values with standard deviations (error bars) from at least three repeats are shown. (B) Interactions between His_6-RpoS and the T6SS4 promoter in the presence of *E. coli* RNA polymerase core enzyme were analyzed using EMSA. As negative controls, only 80 nM His_6-RpoS or 20 nM RNA polymerase core enzyme was added to the reaction mixture (lane 2 or 3); a 700 bp DNA fragment amplified with primers *rpoS*-F697 and *rpoS*-R697 from the *rpoS* gene coding region was used instead of the T6SS4 promoter as a further control (lane 5). (C-D) Survival rates of *Y. pseudotuberculosis* wild-type, Δ*rpoS* mutant, Δ*clpV4* mutant, Δ*rpoS*Δ*clpV4* double mutant and the complementary strains Δ*rpoS*Δ*clpV4*(*rpoS*) and Δ*rpoS*Δ*clpV4*(*clpV4*) after exposure to pH 4.0 (C) or 0.5 M NaCl (D). All experiments were performed independently in triplicate. Error bars are standard deviations of the mean of three replicates. * $P \leq 0.05$ or ** $P \leq 0.01$.

role in T6SS4 activation.

Because RpoS activates T6SS4 expression and T6SS4 has broad effects in stress resistance (Gueguen *et al.*, 2013; Zhang *et al.*, 2013) we further investigated the role of T6SS4 in RpoS-mediated stress resistance in *Y. pseudotuberculosis*. The survival rates of the wild-type, Δ*rpoS* or Δ*clpV4* (a T6SS4 deficient mutant) single mutant, Δ*rpoS*Δ*clpV4* double mutant and the corresponding complementary strains were determined under acid (pH 4.0) and osmotic (0.5 M NaCl) stresses. Unlike the Δ*ompR* mutant, which was more severely affected than the T6SS4 mutant under osmotic and acid stresses (Zhang *et al.*, 2013), the Δ*rpoS* mutant showed a similar survival rate to the Δ*clpV4* mutant, and the survival rate of the Δ*rpoS*Δ*clpV4* double mutant was significantly lower than that of the Δ*rpoS* or Δ*clpV4* single mutant (Fig. 3C and D). These results suggest that T6SS4 plays a role in stress resistance even in the absence of RpoS. Additionally, these results imply that T6SS4 is only partly responsible for the stress-resistance activity of RpoS, consistent with the relatively weak role of RpoS in activating T6SS4 expression. Consistently, the hypersensitivity phenotype of the Δ*rpoS*Δ*clpV4* double mutant was partially recovered in the complementary strains Δ*rpoS*Δ*clpV4*(*clpV4*) and Δ*rpoS*Δ*clpV4*(*rpoS*) (Fig. 3C and D).

Effects of RpoS on bacterial motility and flagellum formation

The flagellum is crucial for the motility of bacteria, mediating their movement towards favorable environments and avoiding unfavorable conditions, and functions as a potential virulence factor for pathogenic bacteria during host infection (Xu *et al.*, 2014b). To determine if the *rpoS* gene affects bacterial motility, swimming motility by the *Y. pseudotuberculosis* wild-type, Δ*rpoS* mutant and the complementary strain Δ*rpoS*(*rpoS*) in semi-solid medium was evaluated. Flagellum-based motility was dramatically reduced in the Δ*rpoS* mutant compared with the wild-type strain and was restored to the wild-type level in the complementary strain Δ*rpoS*(*rpoS*) (Fig. 4A). Flagella morphology was used to study the formation of flagella in the *Y. pseudotuberculosis* wild-type, Δ*rpoS* mutant and the complementary strain Δ*rpoS* (*rpoS*). Cells were cultivated on semi-solid medium, negatively stained with sodium phosphotungstate and then observed using a transmission electron microscope. The Δ*rpoS* mutant produced almost no flagella, while the complementary strain produced flagella similar to the wild type (Fig. 4B).

$FlhD_4C_2$, the product of the flagellar master regulatory gene *flhDC*, is the transcriptional activator complex for the genes downstream in the flagella transcriptional hierarchy (Young *et al.*, 1999). Because the Δ*rpoS* mutant strain produced almost no flagellar filaments, we speculated that RpoS might influence *flhDC* expression. To verify this hypothesis, *flhDC* promoter activities expressed in the *Y. pseudotuberculosis* wild-type, Δ*rpoS* mutant, and the complementary strain Δ*rpoS* (*rpoS*) were examined by using the transcriptional *flhDCp::lacZ* chromosomal fusion reporter. As expected, *flhDC* expression in the Δ*rpoS* mutant was significantly lower than in the wild-type and the complementary strain (Fig. 4C). These data suggest that RpoS activates motility and flagellar formation in *Y. pseudotuberculosis* by controlling *flhDC* expression. This conclusion was further supported by the finding that *flhDC* overexpression in the Δ*rpoS* mutant completely restored the flagellar and motility phenotypes of the mutant to the wild-type level (Fig. 4A and B). However, RpoS regulation of *flhDC* appears indirect, based on our further analysis using the EMSA assay, since RpoS could not bind to the *flhDC* promoter (Supplementary data Fig. S3). Taken together, these results suggest that RpoS plays a central role in flagellar formation and motility by regulating *flhDC* expression, possibly in an indirect manner.

RpoS affects biofilm formation

Flagellum-mediated motility is crucial for specific stages of biofilm formation (Tribelli *et al.*, 2013; Svensson *et al.*, 2014); thus, we assessed the role of RpoS in biofilm formation. The nematode *C. elegans* was used as an ideal biotic surface to evaluate biofilm formation by *Y. pseudotuberculosis*, because *Y. pseudotuberculosis* can form biofilms only on biotic surfaces (Joshua, 2003). Cells of the *Y. pseudotuberculosis* wild-type, Δ*rpoS* mutant and the complementary strain were labeled with GFP by introduction of the constitutive *gfp*-plasmid pKEN-GFP mutant3*. After infection by these strains for 18–24 h, biofilm formation on the nematodes was determined. The severity of biofilm formation was ranked on a scale of 0 to 3, with 0 indicating the lowest level and 3 the highest (Atkinson *et al.*, 2011). The results showed that Δ*rpoS* cells had a higher proportion of class 0 level biofilm severity than did the wild-type and the complementary strain, sug-

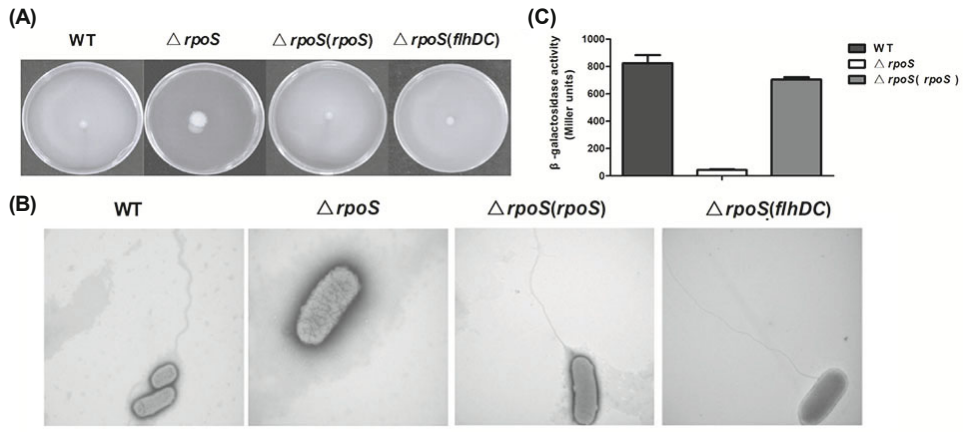

Fig. 4. Impacts of RpoS on motility and *flhDC* promoter activity. (A) Motility of the *Y. pseudotuberculosis* wild-type, Δ*rpoS* mutant, Δ*rpoS*(*rpoS*) and Δ*rpoS*(*flhDC*) on semi-solid plates. (B) Transmission electron micrographs of flagella of the wild-type (WT), Δ*rpoS* mutant, Δ*rpoS* (*rpoS*), and Δ*rpoS*(*flhDC*) strains. (C) β-Galactosidase assay of the effects of RpoS on *flhDC* promoter activity.

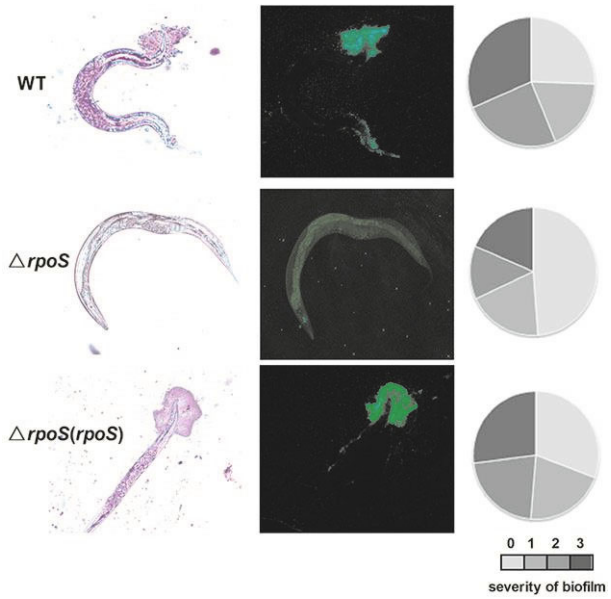

Fig. 5. Effect of RpoS on biofilm formation in *C. elegans*. GFP-labeled *Y. pseudotuberculosis* strains were used to detect biofilm formation. The first vertical columns are bright-field images. The second columns are fluorescence images. The intensity of biofilm formation by the *Y. pseudotuberculosis* wild-type, Δ*rpoS* mutant and the complementary strain Δ*rpoS*(*rpoS*) is shown in the third column, and is classified into four levels; the intensity of biofilm formation increased from 0 to 3.

gesting that fewer nematodes were infected, and less biofilm was formed, by the Δ*rpoS* strain than the wild-type and the complementary strain. Furthermore, the ratios of level 2 and level 3 biofilm severity in Δ*rpoS* were lower than those of the wild-type and the complementary strain (Fig. 5). These results indicate that RpoS positively regulates biofilm formation by *Y. pseudotuberculosis*.

RpoS positively controls synthesis of extracellular polysaccharides (EPS)

The extracellular matrix (ECM) is composed of water, polysaccharides, nucleic acids, proteins and phospholipids and is an important component of biofilms. Among these components, extracellular polysaccharides (EPS) are crucial for biofilm formation (Drace and Darby, 2008). Thus, we determined whether RpoS influences biofilm formation by controlling the synthesis of EPS. To investigate the effect of RpoS on EPS synthesis, exopolysaccharides were purified from culture supernatants by cetyl trimethyl ammonium bromide (CTAB) precipitation and quantified using phenol-vitriol assay (Doherty *et al.*, 1988). Under the function of vitriol, polysaccharides become furfural derivatives and then form a colored complex with phenol, which can be measured by recording the absorbance at 490 nm using a spectrophotometer. As shown in Fig. 6A, the polysaccharide level was significantly lower in the Δ*rpoS* mutant compared with the wild-type and the complementary strain, indicating that RpoS positively modulates exopolysaccharide synthesis in *Y. pseudotuberculosis*.

In *Yersinia* spp., biofilm formation requires *hmsHFRS*, which is homologous to *pgaABCD* and encodes predicted polysaccharide biosynthesis proteins (Drace and Darby, 2008). We next investigated whether RpoS controls polysaccharide synthesis by regulating *hmsHFRS* expression. β-Galactosidase activity assay results showed that in the Δ*rpoS* mutant, the activity of the *hmsHFRS* promoter decreased significantly compared with the wild-type strain. Additionally, the reduction was recovered in the complementary strain (Fig. 6B). These results indicate that RpoS can positively regulate expression of *hmsHFRS*, which encodes polysaccharide biosynthesis proteins. However, the effect of RpoS on *hmsHFRS* expression appears to be indirect based on the EMSA analysis (Supplementary data Fig. S4).

Discussion

Since the discovery of RpoS, it has been characterized as an alternative σ factor responsible for the regulation of so-called general stress response genes that facilitated cell survival under unfavorable conditions. Early studies have shown that RpoS was not essential for growth, but *rpoS* mutant strains were extremely sensitive to various environmental stresses (Loewen and Hengge-Aronis, 1994). Herein, we consistently found that in *Y. pseudotuberculosis*, *rpoS* mutation led to decreased survival rates in the presence of oxidative stress, acid stress, osmotic stress and 42°C heat shock. As expected, *rpoS* deletion in *Y. pseudotuberculosis* resulted in decreased expression of the majority of RpoS-dependent stress response genes. Interestingly, we also found that T6SS4–which

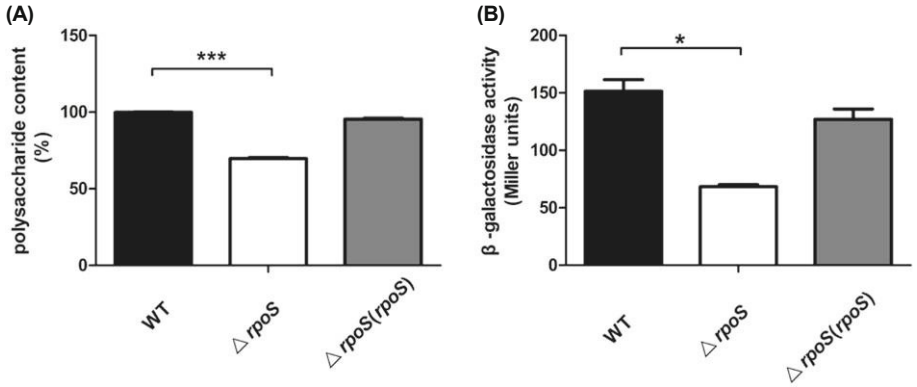

Fig. 6. RpoS positively controls EPS synthesis by regulating *hmsHFRS* expression. (A) Extracellular polysaccharides (EPS) contents in the *Y. pseudotuberculosis* wild-type, Δ*rpoS* mutant and the complementary strain. To compare the polysaccharide contents among strains, the level of polysaccharides in the wild-type was considered as 100%. (B) β-Galactosidase assay of the effect of RpoS on *hmsHFRS* promoter activity.

is involved in stress resistance in *Y. pseudotuberculosis*–was directly activated by RpoS, implicating it in the RpoS-mediated stress resistance.

Accumulating data indicate that T6SS is involved in stress resistance in various bacteria (Weber *et al.*, 2009; Goldova *et al.*, 2011; Records, 2011; Gueguen *et al.*, 2013; Zhang *et al.*, 2013). The finding that RpoS is the key regulator of the stress response and the function of the T6SS under stress conditions prompted us to examine whether RpoS regulates the expression of the stress-resistance T6SS4 in *Y. pseudotuberculosis*. Consistent with our hypothesis, RpoS was found to positively regulate T6SS4 expression. Furthermore, we found that RpoS can bind to the T6SS4 promoter in the presence of the *E. coli* RNA polymerase core enzyme, indicating that the effect of RpoS on the T6SS4 was direct. Previously, Weber proposed that the *V. anguillarum* T6SS plays a role as a sensor for an unknown extracytoplasmic signal that modulates RpoS expression and the stress response (Weber *et al.*, 2009). Our results demonstrated that RpoS could also directly activate T6SS4 expression, suggesting that besides sensing the extracytoplasmic signal, T6SS4 may also act as a general stress response system similar to many other RpoS-depended general stress-response genes, such as *uspB* and *katE*. This hypothesis was supported by the finding that under stress conditions, the Δ*rpoS*Δ*clpV4* double-mutant cells were more severely affected than the Δ*rpoS* single-mutant cells, suggesting that T6SS4 exhibits stress-resistance activity even in the absence of RpoS. Further studies are needed to reveal the mechanisms underlying the role of T6SS in stress resistance.

Because RpoS is reported to participate in the regulation of virulence in several pathogens (Dong and Schellhorn, 2010), we further analyzed its role in the regulation of motility, an important virulence factor. The *Y. pseudotuberculosis rpoS* mutant exhibited significantly reduced motility than the wild-type strain, consistent with the findings in *V. vulnificus* or *P. aeruginosa* (Dong and Schellhorn, 2010). We further demonstrated that the positive control of motility in *Y. pseudotuberculosis* is through the regulation of flagellum expression. RpoS has different effects in regulating flagella expression in various bacterial taxa. In *E. coli*, RpoS represses the expression of *fliA* and thus negatively controls flagellum production (Dong and Schellhorn, 2009). In *P. aeruginosa*, RpoS positively regulates the expression of the flagellar gene *fliF* as well as chemotaxis genes (Schuster *et al.*, 2004). In contrast, we found that in *Y. pseudotuberculosis*, RpoS positively regulates *flhDC*, the flagellum master regulatory gene. However, this is contrary to an earlier report that RpoS negatively regulates *flhDC* expression in acidic phospholipid-deficient *E. coli* (Uchiyama *et al.*, 2010). Since the regulation of *flhDC* in *E. coli* was determined during the mid-exponential growth phase while in this study the regulation of *flhDC* was assayed during the stationary phase, we speculate that the RpoS-mediated regulation of *flhDC* expression may differ according to growth phase.

Numerous Gram-negative bacteria depend on flagellum-mediated motility for specific stages of biofilm formation (Tribelli *et al.*, 2013). Therefore, we investigated the contribution of RpoS to biofilm formation on *C. elegans*. Indeed, RpoS positively regulated *Y. pseudotuberculosis* biofilm formation (Fig. 5), suggesting that RpoS regulates the expression of potential virulence factors that may be responsible for *Y. pseudotuberculosis* pathogenicity. In *E. coli*, five sets of genes promote EPS synthesis–the *wca* operon, *pgaABCD* operon, *dfc* pseudo operon, *yjbEFGH* operon and O-antigen synthesis related genes (Gottesman *et al.*, 1985; Wang *et al.*, 2004; Peleg *et al.*, 2005). Among these, the *pgaABCD* operon encodes genes involved in the production of poly-1,6-*N*-acetyl-D-glucosamine (PGA), a polysaccharide crucial for biofilm formation. In contrast, in *Y. pseudotuberculosis*, the *hmsHFRS* operon, which is homologous to the *pgaABCD* operon, is responsible for the biosynthesis of the exopolysaccharide poly β-1,6-N-acetyl-D-glucosamine-like polysaccharide, which plays an indispensable role in biofilm accumulation on *C. elegans*. Additionally, *hmsHFRS* mutants of *Y. pseudotuberculosis* failed to form biofilms on *C. elegans* (Drace and Darby, 2008). Therefore, we determined whether RpoS regulates biofilm formation by modulating *hmsHFRS* expression. RpoS positively regulated *hmsHFRS* expression, albeit indirectly (Fig. 6). The *rpoS* mutant exhibited reduced *hmsHFRS* transcription and EPS production. To our knowledge, this is the first report that RpoS regulates *hmsHFRS* expression, thus affecting the synthesis of poly-1,6-*N*-acetyl-D-glucosamine (PGA), and influences biofilm formation.

However, the role of RpoS in biofilm development remains controversial. For example, in *E. coli*, an RpoS knockout mutant produced less biofilm than the wild type (Adams and McLean, 1999). Conversely, another study showed that *P. aeruginosa* and *E. coli* Δ*rpoS* mutants exhibited enhanced biofilm formation than the wild-type strains (Whiteley *et al.*, 2001; Corona-Izquierdo and Membrillo-Hernandez, 2002). However, formation of biofilm is a complicated process that can be influenced by other proteins, which may explain the discrepant results. For example, in *Y. enterocolitica*, OmpR can promote biofilm formation, although additional OmpR-dependent factors are also required. In addition, the study suggested that regulation of biofilm formation could be an additional aspect of the regulatory function of OmpR (Raczkowska *et al.*, 2011). A better understanding of the regulation of biofilm formation and the expression of stress response genes could lead to discovery of novel pathways in bacterial pathogens.

In summary, our study results indicated that in addition to its contribution to bacterial survival under various stress conditions, RpoS is necessary for the regulation of virulence factors–including flagella and biofilm formation–in *Y. pseudotuberculosis*. These results strongly suggest that as an enteropathogenic bacterium, *Y. pseudotuberculosis* can survive in adverse environments and then cause disease, with the assistance of RpoS. Therefore, RpoS may serve as a target for designing novel antibacterial drugs, and the RpoS mutant as a vaccine candidate for preventing intestinal diseases caused by *Y. pseudotuberculosis*.

Competing interests

The authors declare that they have no competing interests.

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