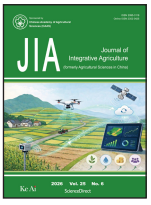
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RESEARCH ARTICLE

(p)ppGpp interacts with TrmE and regulates the PcoI/PcoR quorum-sensing system of *Pseudomonas fluorescens* 2P24

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Highlights

- RelA and SpoT exert opposite effects on the PcoI/PcoR quorum-sensing (QS) system in *Pseudomonas fluorescens* 2P24.
- (p)ppGpp regulates the PcoI/PcoR QS system by influncing the function of the tRNA modification GTPase TrmE.

Abstract

The efficient colonization of plant-beneficial *Pseudomonas* spp. is a prerequisite for their biocontrol capacity. Prior work revealed that the PcoI/PcoR quorum-sensing (QS) system plays a pivotal role in the root colonization of *P. fluorescens* 2P24. During the colonization, strain 2P24 has faced diverse impacts from plant-derived reactive oxygen species and other environmental stress. However, the molecular mechanism by which the PcoI/PcoR QS system is regulated under unfavored conditions remains unclear. Thus, in this study, the role of the (p)ppGpp synthetase RelA and the bifunctional (p)ppGpp synthase/hydrolase SpoT in the PcoI/PcoR QS system of *P. fluorescens* was investigated. Our data indicated that the deficiency of *relA* and *spoT* genes remarkably improved the expression of the *pcoI* gene, whereas the mutation of the *spoT* gene significantly repressed the expression of the *pcoI* gene. We further demonstrated that the regulation of the PcoI/PcoR QS system by (p)ppGpp was dependent on the function of the *trmE* gene, which encodes a tRNA modification GTPase. Furthermore, the mutation of *relA*, *spoT*, or both significantly influenced the motility, biofilm formation, oxidative stress, osmotic tolerance, and rhizosphere colonization. Collectively, our data indicated that the (p)ppGpp signaling pathway mediated by the *relA* gene and *spoT* gene was important to the function of the PcoI/PcoR QS system and had important implications for the understanding of the molecular mechanism of (p)ppGpp in epiphytic fitness *via* TrmE of *P. fluorescens*.

Keywords: *Pseudomonas fluorescens*, quorum sensing, *relA*, *spoT*, *trmE*

1. Introduction

Rhizospheric plant-beneficial *Pseudomonas* spp. utilize the exudates secreted by plant roots to survival, produce diverse microbial secondary metabolites, and induce systemic acquired resistance to protect plant health. In addition, this plant–microbe interaction is beneficial for soil microbial diversity and sustainable agriculture (Kang *et al*. 2021). However, the successful establishment of plant–microbe symbiosis mainly relies on the efficient rhizosphere colonization of these rhizobacteria. Rhizosphere colonization is a highly coordinated process in which bacteria need to undergo multiple strategies to compete with neighboring microorganisms under adverse environmental conditions. Several studies have reported that many biotic and abiotic factors influenced rhizosphere colonization (Santoyo *et al*. 2021). Certain plants were shown to specifically recruit phytobeneficial rhizobacteria to colonize through the production of selectively compounds in their exudates (Pomerleau *et al*. 2024). Extracellular bacterial structures such as flagella, fimbriae, and the O antigen of lipopolysaccharide could regulate the attachment of bacterial cells to the root surface and then influence the efficiency of root colonization (Mavrodi *et al*. 2006). Moreover, root colonization is regulated by many genetic elements.

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The genes responsible for the metabolite biosynthesis of vitamin B1 (Simons *et al.* 1997), the CoIR/CoIS two-component regulatory system (Dekkers *et al.* 1998), the ABC putrescine transport system (Kuiper *et al.* 2001), the phosphoenolpyruvate-protein phosphotransferase PtsP (Mavrodi *et al.* 2006), and the sensor kinase KinB (Barahona *et al.* 2011) have been shown to enhance or impair the root colonization in some *Pseudomonas* spp. strains. Although many beneficial rhizobacteria show strong and reproducible biocontrol capacity in controlled conditions, the root colonization effects are variable in field conditions. To data, little is known about the molecular processes by which root colonization is influenced under distinct environmental stresses, particularly of nutrient starvation.

The stringent response is a critical stress signaling system that enables bacteria to respond to nutrient deprivation. This process is regulated by two intracellular signaling molecules, namely, guanosine pentaphosphate and guanosine tetraphosphate (collectively referred to as (p)ppGpp) (Dalebroux *et al.* 2010). In bacteria, the RelA-SpoT homologue (RSH) proteins are involved in the synthesis and degradation of (p)ppGpp (Atkinson *et al.* 2011). Under amino acid limited conditions, RelA directly interacts with ribosome and synthesizes (p)ppGpp when the ribosome stalls due to the binding of unchanged tRNA in the A sites. The bifunctional SpoT, which has both hydrolase and synthase activities, could be activated under adverse conditions (Magnusson *et al.* 2005). Apart from nutrient scavenging, recent studies have shown that (p)ppGpp plays a central role in bacterial pathogenesis, host invasion, antibiotic tolerance, nitrogen fixation and swimming motility (Irving *et al.* 2021). In *Escherichia coli* and many γ-proteobacteria, (p)ppGpp mainly exerts its physiological roles through direct or indirect mechanisms. The direct mechanism of (p)ppGpp is associated with RNA polymerase (RNAP) and the transcriptional regulator DksA (Dalebroux and Swanson 2012). (p)ppGpp can directly bind to RNAP in cooperation with DksA, and this interaction results in a synergistic role in the targeted gene expression. Interestingly, DksA also binds to RNAP without (p)ppGpp. Therefore, (p)ppGpp and DksA could play independent divergent roles in many phenotypes, such as growth and biofilm formation (Huang *et al.* 2022). The indirect mechanism of (p)ppGpp requires intracellular enzymes or transcription factors (Zhang *et al.* 2018a). In *Bacillus subtilis*, the dissociation of the transcriptional repressor CodY to the DNA occurs because GTP pools drop as a result of (p)ppGpp synthesis when cells experience nutrient deprivation (Belitsky and Sonenshein 2011). Besides DksA, translational initiation factor 2 (IF2) was reported to directly bind (p)ppGpp and this interaction between (p)ppGpp and IF2 could influence the IF2-dependent initiation complex formation and then hinder the initiation step of translation (Milon *et al.* 2006). In addition, many GTPases, such as RsgA, EF-G, and EngB were identified as (p)ppGpp-binding proteins (Mitkevich *et al.* 2010; Wang *et al.* 2019).

Although the function of (p)ppGpp is widely studied, whether (p)ppGpp participates in the regulation of the rhizosphere colonization and biocontrol capacity of plant-beneficial *Pseudomonas* spp. still needs to be explored. *P. fluorescens* 2P24 is an effective biocontrol rhizobacterium, which can control several soilborne diseases by efficient colonization of plant roots and producing some secondary metabolites (Wei *et al.* 2004). In a previous study, the depletion of the PcoI/PcoR quorum-sensing (QS) system impaired the establishment of *P. fluorescens* on the roots of wheat, indicating that the PcoI/PcoR QS is involved in the root colonization of strain 2P24 (Yu *et al.* 2022). The QS system is a cell-cell communication system to coordinate population density dependent social behaviors. This system relies on the production and sensing of low-molecular-weight molecules as intercellular signals. The best-studied signals of the QS system in Gram-negative bacteria are *N*-acylhomoserine lactones (AHLs). In strain 2P24, the PcoI/PcoR QS system consists of the QS signal synthase PcoI and its cognate regulator PcoR (Wei and Zhang 2006). Recent studies have shown that the expression of *pcoI* is positively regulated by PcoR by directly binding to the promoter region of *pcoI*. In addition, the repressor RsaL binds simultaneously with PcoR to the promoter region of *pcoI*, and fine tuning regulates the AHL pools in the cells (Yu *et al.* 2022). The PcoI/PcoR QS system is also regulated by the Gac/Rsm regulatory cascade involves the GacS/GacA two-component regulatory system, small non-coding RNAs (sRNA), and its cognate RNA-binding proteins RsmA and RsmE (Zhang *et al.* 2020).

In the present study, the effects of the *relA* gene and the *spoT* gene on the PcoI/PcoR QS system in strain 2P24 were examined. Our findings indicated that the modulation of the concentration of (p)ppGpp in the cells remarkably influenced the PcoI/PcoR QS system. Microscale thermophoresis and β-galactosidase activity assays revealed that (p)ppGpp could regulate the PcoI/PcoR QS system by directly interacting with tRNA modified GTPase TrmE. Moreover, the important roles of *relA* and *spoT* in the regulation of general stress resistance, motility, biofilm formation, and root colonization were reported. Overall, our study not only uncovers that the PcoI/PcoR QS system is regulated by (p)ppGpp, but also sheds light on the mechanism of (p)ppGpp in epiphytic fitness *via* TrmE of *P. fluorescens*.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Escherichia coli strain was routinely cultured at 37°C in lysogeny broth (LB) medium, whereas LB, KB (King *et al.* 1954), or M9 medium (Miller 1972) was used for *P. fluorescens* 2P24 and its derivative. *Agrobacterium tumefaciens* NTL4 (pZLR4) was cultured at 28°C in ABM medium (Chilton *et al.* 1974) with 40 μg mL^{-1} of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) when required. The strains and plasmids used in this study are listed in Appendix A. Antibiotics were added to the medium as necessary: ampicillin (50 μg mL^{-1}), kanamycin (50 μg mL^{-1}), tetracycline (20 μg mL^{-1}) were added.

2.2. Strain and plasmid construction

In-frame deletions were achieved using *sacB*-based allelic exchanged as previously described (Zhang *et al.* 2018b).

Briefly, upstream and downstream regions flanking the target gene were amplified by PCR (primers are listed in Appendix B) and ligated into the suicide vector p2P24Km (Zhang *et al.* 2018b). In frame-deletion constructs were directly electroporated into *P. fluorescens*. The insertion mutants were selected by antibiotic resistance and double recombinants were obtained using sucrose counter-selection. The successful deletion was screened by PCR using flanking primers and verified by Sanger sequencing (Sangon, Shanghai, China). Complementation plasmids were generated with pRK415 plasmid (Keen *et al.* 1988), which were introduced into *P. fluorescens* by electroporation.

To create the (p)ppGpp reporter plasmid (p6013-ppGpp), the YkkC riboswitch encoding region were PCR amplified using *B. subtilis* 1A234 genomic DNA (Sherlock *et al.* 2018) as the template, and the resulting fragment was ligated into the pME6013 between the *Bam*HI and *Pst*I sites, which harbored a promoterless *lacZ* gene (Schnider-Keel *et al.* 2000).

Mutations were introduced in the conserved motif of TrmE by using the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies, USA) according to the manufacturer's protocol, using p415-trmE as the template.

2.3. The β-galactosidase activity assay

Pseudomonas fluorescens 2P24 and its derivatives containing p6013-ppGpp, p970Km-oxyRp, or p970Km-katBp were grown at 30°C in 50-mL flasks containing 20 mL of LB medium (Yang *et al.* 2023). Cells were collected and the β-galactosidase activities were quantified using the Miller method (Miller 1972).

2.4. Isolation and detection of AHLs

Pseudomonas fluorescens cells were cultured in 5 mL LB broth at 30°C for 18 h. The culture supernatants were collected and then extracted with an equal volume of ethyl acetate. The extracts were dried and resuspended in 50 mL methanol. An aliquot (5 mL) of each extract was loaded onto the ABM medium seeded with *A. tumefaciens* NTL4 (pZLR4) and 40 μg mL^{-1} X-gal. The plates were incubated at 30°C for 12 h, and the production of AHLs was determined by the formation of a blue zone around the sample. Three biological replicates were used, and each was analyzed in three technical replicates.

2.5. Immunoblot analyses

Pseudomonas fluorescens cells were cultured overnight in LB broth, diluted 1:1,000, and incubated at 30°C for 6 h. Cells were harvested and the pellets were resuspended in the loading buffer. Samples from equivalent numbers of bacterial cells were loaded and separated on a 10% SDS-PAGE gel. The proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, USA). FLAG-tagged PcoR proteins on the PVDF membrane were hybridized with an anti-FLAG mouse monoclonal antibody (TransGen Biotech, China). The horseradish peroxidase (HRP)-goat anti-mouse IgG secondary antibody was used for chemiluminescent detection of bound ligands by the use of a chemiluminescence detection kit (Thermo Fisher, USA). RNA polymerase beta (RNAP) was used as the reference protein as the internal standard (Thermo Fisher, USA). The intensities of the resulting bands were analyzed with ImageJ software (Schneider *et al.* 2012). The experiment was repeated three times with three replicates in each assay.

2.6. Microscale thermophoresis (MST) assay

MST was performed to analyze the interaction between (p)ppGpp and TrmE. The *trmE* gene was cloned into pET21b and the fusion protein TrmE-His was induced and purified as described previously (Yu *et al.* 2022). The purified TrmE-His was labelled with the fluorescent dye NT-647-NHS (NanoTemper Technologies) *via* amine conjugation. A constant concentration (500 μmol L^{-1}) of the labelled target protein in MST buffer (50 mmol L^{-1} Tris, pH 7.5, 150 mmol L^{-1} NaCl, 10 mmol L^{-1} $MgCl_2$, 0.05% Tween 20) was titrated against increasing concentrations of (p)ppGpp, which were dissolved in diethylpyrocarbonate-treated water. MST premium-coated capillaries (Monolith NT.115 MO-K025, Germany) were used to load the samples into the MST instrument at 25°C using 80% MST power and 20% LED power. The dissociation constant (K_d) was calculated using Nanotemper Analysis v2.3 software (NanoTemper Technologies GmbH, Munich, Germany). The experiment was repeated three times with three replicates in each assay.

2.7. Bacterial growth

Pseudomonas fluorescens cultures grown overnight in LB medium were diluted 1:100 in LB medium, and the bacterial growth was then monitored by measuring the optical density at 600 nm, and presented as the mean standard deviations. The experiment was repeated three times with three replicates in each assay.

2.8. H_2O_2 killing assay

H_2O_2 killing assay was performed as described previously (Yang *et al.* 2023). Briefly, *P. fluorescens* cells were grown at 30°C for 18 h in LB medium, and 5 mL cultures were then added to 100 mL of preheated LB agar (50°C), mixed and poured into the Petri dish. A 5-mm diameter sterile filter paper disk impregnated with 10 mL H_2O_2 at concentration of 1 mol L^{-1} was placed on the top of the LB agar after the plates were solidified. The diameter of the zone of growth inhibition around each disk was determined after 10 h of incubated at 30°C. The experiment was repeated three times with three replicates in each assay.

2.9. Motility assays

The medium used for the motility assay was LB broth containing 0.3% agar for swimming motility and 0.5% agar for swarming motility. Overnight bacterial cultures were washed twice with phosphate-buffered saline (PBS) and diluted to final OD_{600} of 1.0. A total of 5 μL of bacterial suspension was directly inoculated at the center of an agar plate. The plates were incubated at 30°C for 24 h and the diameter of the motility zone was then measured. Every assay was performed three times with six replicates in each experiment.

2.10. Biofilm formation

The crystal violet binding assay were performed to evaluate the formation of biofilm (Wei and Zhang 2006). *Pseudomonas fluorescens* cells were grown overnight in LB broth with at 180 r min^{-1}, diluted 1:100, and incubated in a 96-well polystyrene plate and incubated at 30°C for 24 h without shaking. Then, 50 μL of 0.1% crystal violet solution was added to each well to stain biofilms for 10 min. After a brief wash, the crystal violet was dissolved in 200 μL of 95% ethanol, and the absorbance at 570 nm was detected to quantify biofilm mass. The experiment was repeated three times with six replicates in each assay.

2.11. Root colonization assay

Pseudomonas fluorescens 2P24 and its derivatives were labeled by streptomycin sulfate resistance (Weller 1983). Surface-sterilized wheat seeds were dipped with the bacterial suspension (10^8 CFU mL^{-1}) for 10 min as described previously (Liang *et al.* 2020). Then, eight wheat seeds were planted into a pot containing sterile soil. After 7 days, the root tip was isolated and bacteria were removed from the root by vertexing. Subsequently, the number of CFU was determined by dilution plating. The experiment was repeated three times, and population data were $\log_{10}$ transformed before statistical analysis.

2.12. Bioinformatics analysis

Amino acid alignment of the TrmE homolog was aligned by use of Clustal Omega multiple sequence alignment tool on EMBL-EBI website (https://www.ebi.ac.uk). The amino acid sequences of TrmE were submitted to the online server Swiss-Model (https://swissmodel.expasy.org/) to construct three-dimensional structures and 1XZP (PDB ID) was employed as the template.

2.13. Statistical analysis

GraphPad Prism 8 was used for all analyses. Analysis of variance (ANOVA) was used to determine significance with Tukey's multiple comparisons tests. A *P*-value of <0.05 was considered to be significant.

3. Results

3.1. *spoT* gene and *relA* gene influence the PcoI/PcoR QS system

The (p)ppGpp-mediated stringent response is an important signal transduction system that enables bacteria to coordinate their cellular processes in response to environmental stresses. To determine the role of stringent response in biocontrol traits of *P. fluorescens* 2P24, the *relA* mutant, *spoT* mutant, and *relA spoT* double mutant were generated. The relative intracellular (p)ppGpp level was analyzed using a (p)ppGpp reporter, which contains a transcriptional fusion of β-galactosidase (encoded by the *lacZ* gene) to the *YkkC* RNA riboswitch, a high-affinity (p)ppGpp aptamer (Sherlock *et al.* 2018). The binding of (p)ppGpp to the *YkkC* riboswitch induced *lacZ* expression; therefore, a high (p)ppGpp pool would lead to a high level of β-galactosidase activity. The results indicated that the β-galactosidase activity of the *relA* mutant was significantly decreased compared with that of the wild-type strain. By contrast, the β-galactosidase activity of the *spoT* mutant was significantly increased compared with that of strain 2P24 (Fig. 1). Furthermore, the mutation of *relA* and *spoT* greatly impaired the β-galactosidase activity level compared with that of the *relA* mutant (Fig. 1). Collectively, these results indicated that *relA* and *spoT* are necessary for the production of (p)ppGpp in strain 2P24.

Then, we determined whether the expression of the PcoI/PcoR QS system is regulated by *relA* or *spoT*. Transcriptional fusion assays showed that no remarkable difference in the expression of *pcoI* between the *relA* mutant and the wild-type, whereas the expression of *pcoI* was significantly increased in the *relA spoT* double mutant compared with that of the wild-type (Fig. 2-A). Furthermore, the expression of *pcoI* was significantly reduced in the *spoT* mutant relative to the wild-type (Fig. 2-A). Similar regulatory trends were observed in the production of AHLs (Fig. 2-B), indicating that (p)ppGpp turnover could influence the PcoI/PcoR QS system of *P. fluorescens*.

Considering that the transcriptional regulator PcoR regulated the expression of *pcoI* by directly binding to the promoter region of *pcoI*, whether this effect resulted from the effect of (p)ppGpp on the expression of *pcoR* was investigated. As expected, the expression of *pcoR* was downregulated in the *relA spoT* double mutant when compared with the wild-type (Fig. 2-C). By contrast, the mutation of the *spoT* gene remarkably increased the expression of *pcoR* (Fig. 2-C). In addition, WB assays were conducted to confirm the effect of (p)ppGpp on the levels of PcoR (Fig. 2-D). Collectively, our data indicated that the *relA* gene and *spoT* gene played a vital role in the function of the PcoI/PcoR QS system.

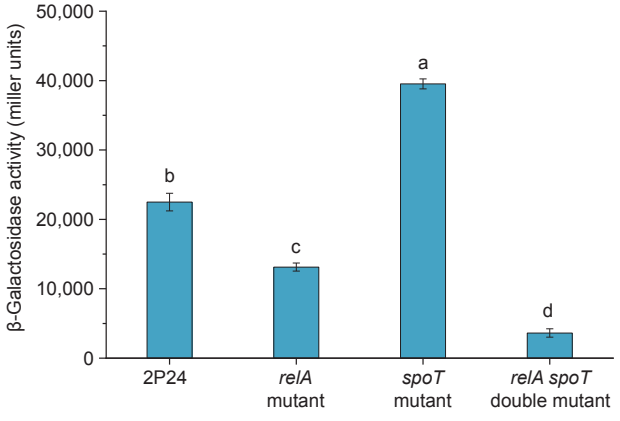


Fig. 1 Relative (p)ppGpp concentrations of *Pseudomonas fluorescens* 2P24, the *relA* mutant, the *spoT* mutant, and the *relA spoT* double mutant. Values were determined by measuring the β-galactosidase activity of the (p)ppGpp riboswitch fusion using the p6013-ppGpp vector. The experiment was performed in triplicate with similar results. Bars indicate one standard deviation from the mean (n=3), while different letters above columns indicate significant differences (P<0.05) according to one-way ANOVA with Tukey's multiple comparisons tests.

3.2. (p)ppGpp controls the PcoI/PcoR QS system in a sRNA, RsaL, or RpoS-independent manner

The Gac/Rsm signal pathway is a key element controlling the QS system of *P. fluorescens* (Zhang *et al.* 2020). To investigate whether (p)ppGpp regulated the QS system through the Gac/Rsm pathway, we examined the promoter activity of sRNA-encoding genes. No difference in the expression of *rsmX*, *rsmX1*, *rsmY*, *rsmZ*, or *rgsA* was observed among the *relA* mutant, *spoT* mutant, *relA spoT* double mutant, and wild-type (Fig. 3-A), indicating that (p)ppGpp influenced the function of the QS system independently of these sRNAs.

Apart from sRNAs, the stationary sigma factor RpoS and the pathway-specific repressor RsaL may regulate the QS system of *P. fluorescens* (Yan *et al.* 2009; Yu *et al.* 2022). As shown in Fig. 3-B and C, the promoter activity of *rpoS* or *rsaL* in the *relA* mutant, *spoT* mutant, or *relA spoT* double mutant was not remarkably changed compared with the wild-type. Therefore, our data showed that the above mentioned regulatory elements were not associated with the regulatory circuit of the PcoI/PcoR QS system mediated by (p)ppGpp.

3.3. DksA1 positively regulates the PcoI/PcoR QS system

Considering that (p)ppGpp could directly bind to RNAP with the assistance of DksA and then regulate the transcription of target genes (Ross *et al.* 2016), we were interested in determining whether DksA was involved in the regulation of the PcoI/PcoR QS system. First, we searched for DksA homologs in *P. fluorescens* using the psi-BLAST algorithm. Interestingly, two *dksA* paralogs were identified in the *P. fluorescens* genome, namely, *dksA1* (C0J56_25295) and *dksA2* (C0J56_12575). Sequence analyses indicated that DksA1 and DksA2 had 64.9 and 23.7% sequence identity, respectively, with DksA of *E. coli* (Fig. 4-A).

To investigate the role of DksA1 and DksA2 in the PcoI/PcoR QS system of *P. fluorescens*, the *dksA1* mutant and *dksA2* mutant were constructed, respectively, and the expression of *pcoI* and the production of AHLs in these mutants were examined. As shown in Fig. 4-B and C, compared with the wild-type, the expression of *pcoI* or the production of AHLs was significantly deceased in the *dksA1* mutant, whereas the expression of *pcoI* and the production of AHLs were unaffected in the *dksA2* mutant, indicating

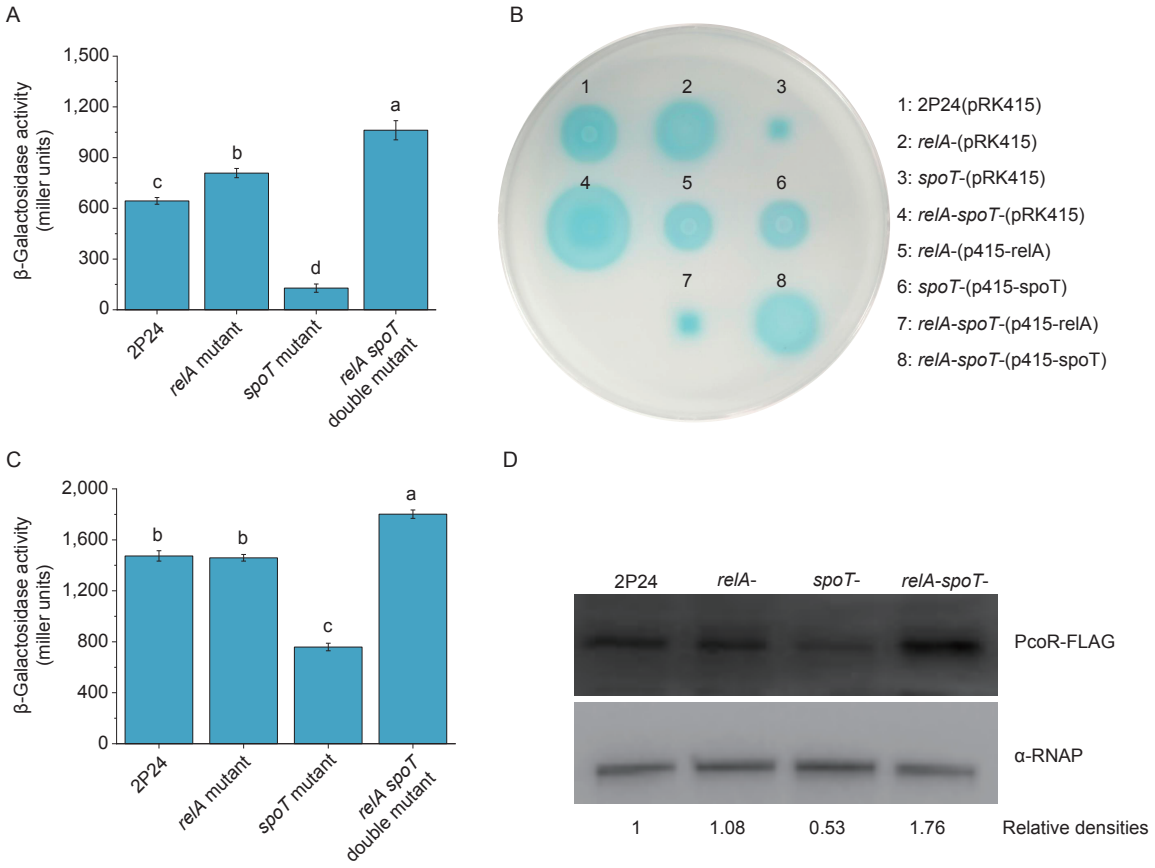


Fig. 2 The *spoT* gene and *relA* gene influences the PcoI/PcoR QS system of *Pseudomonas fluorescens*. A, expression of *pcoI* was regulated by the *relA* and *spoT* genes. The β-galactosidase activity of the transcriptional fusion *pcoI-lacZ* was measured in strain 2P24, the *relA* mutant, the *spoT* mutant, and the *relA spoT* double mutant. B, production of *N*-acylhomoserine lactones (AHLs) of strain 2P24 and its derivatives was determined using the *Agrobacterium tumefaciens* NTL4 (pZLR4) bioreporter. C, the expression of *pcoR* was regulated by the *relA* and *spoT* genes. The β-galactosidase activity of the transcriptional fusion *pcoR-lacZ* was measured in strain 2P24, the *relA* mutant, the *spoT* mutant, and the *relA spoT* double mutant. D, Western blot analysis was performed to detect the levels of PcoR-FLAG in strain 2P24, the *relA* mutant, the *spoT* mutant, and the *relA spoT* double mutant. All experiments were repeated at least three times with similar results. Bars indicate one standard deviation from the mean (n=3), while different letters above columns indicate significant differences (P<0.05) according to one-way ANOVA with Tukey's multiple comparisons tests.

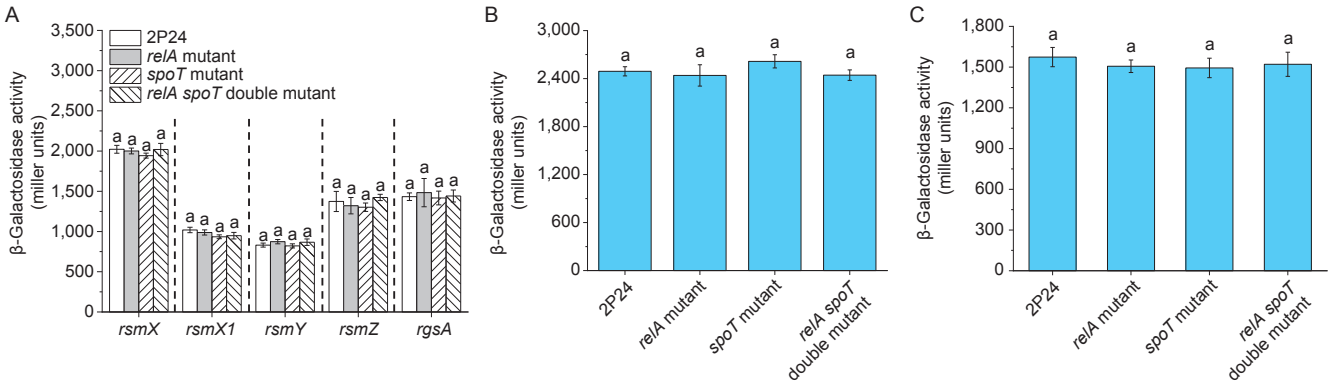


Fig. 3 (p)ppGpp controls the PcoI/PcoR QS system through a sRNAs, RpoS, or RsaL-independent manner. The expression of *rsmX-lacZ*, *rsmX1-lacZ*, *rsmY-lacZ*, *rsmZ-lacZ*, or *rgsA-lacZ* (A), *rpoS-lacZ* (B), and *rsaL-lacZ* (C) transcriptional fusions were determined in strain 2P24, the *relA* muant, the *spoT* mutant, the *relA spoT* double mutant. All experiments were repeated three times with similar results. Bars indicate one standard deviation from the mean (n=3), while different letters above columns indicate significant differences (P<0.05) according to one-way ANOVA with Tukey's multiple comparisons tests.

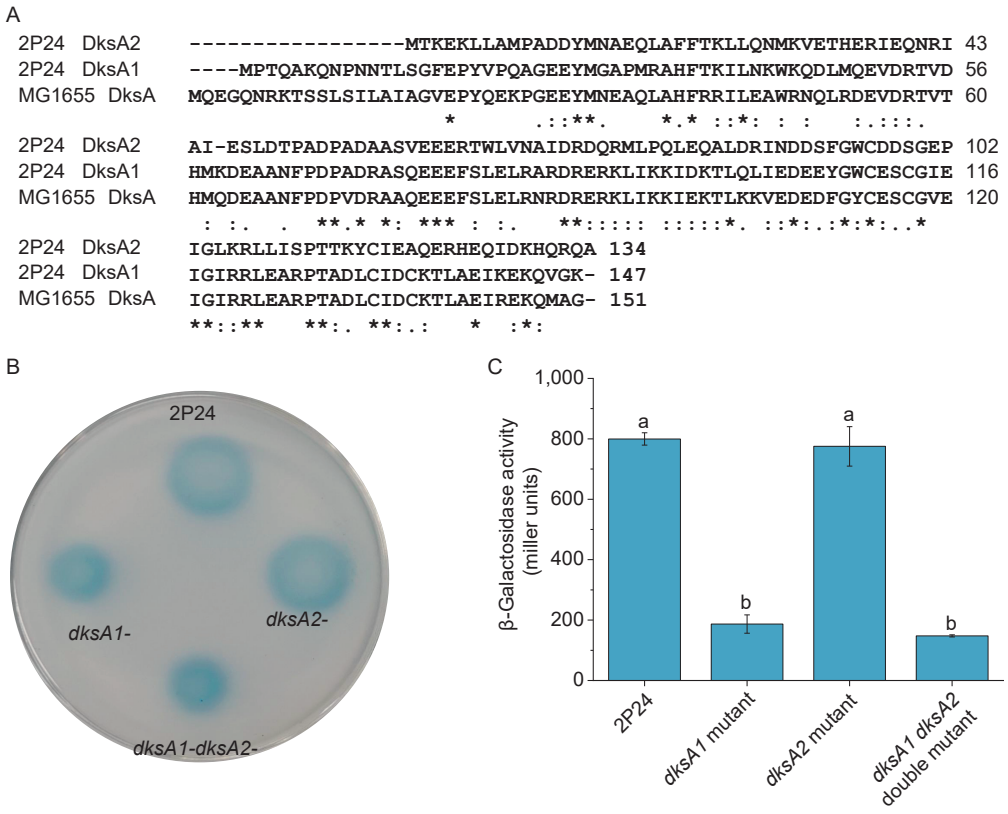


Fig. 4 Effect of the *dskA1* gene and the *dksA2* gene on the PcoI/PcoR QS system of *Pseudomonas fluorescens* 2P24. A, clustal omega multiple sequence alignment of *P. fluorescens* DksA protein with *Escherichia coli* DksA protein. Identical residues (asterisks), conserved residues (colons), and semi-conserved (dots) are indicated. B, production of AHLs of strain 2P24, the *dksA1* mutant, the *dksA2* mutant, and the *dksA1 dksA2* double mutant was determined using the *Agrobacterium tumefaciens* NTL4 (pZLR4) bioreporter. C, β-galactosidase activity of the transcriptional fusion *pcoI-lacZ* was measured in strain 2P24, the *dksA1* mutant, the *dksA2* mutant, and the *dksA1 dksA2* double mutant. The experiments were performed in triplicate with similar results. Bars indicate one standard deviation from the mean (n=3), while different letters above columns indicate significant differences (P<0.05) according to one-way ANOVA with Tukey's multiple comparisons tests.

that *dksA1* positively regulated the PcoI/PcoR QS system. Considering that the mutation of *relA* and *spoT* ((p)ppGpp-deficient) remarkably increased the expression of *pcoI* (Fig. 4), and if (p)ppGpp coordinated with DksA1 to influence the PcoI/PcoR QS system, the mutation of *dksA1* would increase the expression of *pcoI*. However, the expression of *pcoI* was positively regulated in the *dskA1* mutant compared with the wild-type strain (Fig. 4), indicating that the (p)ppGpp-mediated regulation of the PcoI/PcoR QS system is DksA1-independent. Collectively, our data indicated that DksA1 but

not DksA2 was involved in the regulation of the PcoI/PcoR QS system and that (p)ppGpp regulated the PcoI/PcoR QS system independently of DskA1 in *P. fluorescens*.

3.4. Regulation of the PcoI/PcoR QS system by (p)ppGpp requires the *trmE* gene

Previous studies showed that (p)ppGpp regulated many cellular processes by directly interacting with (p)ppGpp-binding proteins, and ~50 proteins such as YgdH, LepA, and PhoA have been reported to bind with (p)ppGpp in *E. coli* (Wang *et al.* 2019). To comprehensively understand the regulatory mechanism of (p)ppGpp in the PcoI/PcoR QS system, the homologues of known (p)ppGpp-binding proteins of *P. fluorescens* were identified using the psi-BLAST algorithm. A total of 16 (p)ppGpp-binding proteins were identified in *P. fluorescens*, based on their physiological functions, which could be classified into five categories, including purine nucleotide biosynthesis, ribosome and translation, gene transcription, (p)ppGpp homeostasis, and dehydrogenase maturation (Appendix C).

Subsequently, the in-frame deletion mutants of the (p)ppGpp-binding protein-encoding genes were constructed to assess whether these 16 candidates mediated the regulation of the PcoI/PcoR QS system. Our data indicated that no difference was observed in the AHL production in *guaB*, *obgE*, *der*, *dnaG*, *mutT*, *purA*, *hflX*, *prfC*, *apt*, *tufB*, *lepA*, *rsgA*, *era*, *gppA*, or *ppnN* mutant was observed compared with that of the wild-type (Appendix D). However, relative to the wild-type, the *trmE* mutant showed a lower production of AHLs (Appendix D; Fig. 5-A). Complementation of the *trmE* mutant by expression of *trmE* in trans restored the production of AHLs to the wild-type level (Fig. 5-A). A similar regulation was observed when the expression of *pcoR* or the levels of PcoR were determined in strain 2P24 and the *trmE* mutant (Fig. 5-B and C). Considering that the conserved nucleotide-binding motif (GxxxxGKS/T, IxxTT, DxxG, and NKxD) is required for the function of TrmE (Scrima *et al.* 2005), we investigated the role of the GxxxxGKS/T motif and the DxxG motif in the regulation of AHL production. The results indicated that the mutation of the GxxxxGKS/T motif (p415-trmEGI) or the DxxG motif (p415-trmEGIII) in TrmE abolished its effect on AHL production (Fig. 5-A). To investigate whether the regulatory pathway of (p)ppGpp was mediated by the *trmE* gene, a *trmE* gene deletion mutant was constructed in the *relA spoT* double mutant background, and the expression of *pcoI* or the production of AHLs in this mutant was examined. As expected, compared with the *trmE* mutant, no detectable impact on the expression of *pcoI* or the production of AHLs was observed when *relA* and *spoT* were deleted in the *trmE* mutant background (Fig. 5-A and D). Next, we determined the interaction of TrmE with (p)ppGpp using MST assay. The MST results showed the binding of (p)ppGpp to TrmE with a calculated binding constant (K_d) of (0.3±0.1) mmol L^{-1} (Fig. 5-E). Collectively, these results indicated that TrmE participated in the regulation of the PcoI/PcoR QS system-mediated by (p)ppGpp.

3.5. Effect of *relA* and *spoT* on the growth of *P. fluorescens*

The growth of the wild-type and the *relA*, *spoT*, and *relA spoT* double mutants was evaluated in liquid cultures in LB medium, KB medium, and M9 minimal medium. No differences in growth curve were observed in LB medium or KB medium (Fig. 6-A and B). However, the *spoT* mutant showed an extended lag phase compared with the wild-type in M9 minimal medium (Fig. 6-C). By contrast, the mutation of the *relA* gene remarkably induced the growth of strain 2P24 in M9 minimal medium. Furthermore, lowering the levels of (p)ppGpp (in the *relA spoT* double mutant) remarkably increased the growth curve of *P. fluorescens* in M9 minimal medium (Fig. 6-C).

To elucidate the potential role of the *relA* gene and *spoT* gene in regulating growth adaptation to nutritional stress, the cell growth of strain 2P24 and its derivatives was determined during amino acid downshift and carbon downshift. The growth transition of strain 2P24 from M9 minimal medium with 0.2% casamino acid to M9 minimal medium was initially examined. Our data showed that the *spoT* mutant had a shorter lag time compared with that of strain 2P24, whereas the lag time of the *relA* mutant or the *relA spoT* double mutant during amino acid downshift was longer (Fig. 6-D). Glucose is the preferred carbon source of *P. fluorescens* (Zhang *et al.* 2018b). In this study, *P. fluorescens* cultures were shifted from glucose to glycerol. During the carbon shift of glycerol, the *spoT* mutant exhibited a shortened growth lag than compared with that of the wild-type. Moreover, the longer lag of the *relA* mutant or the *relA spoT* double mutant than the wild-type was observed during the carbon downshift (Fig. 6-E). Collectively, these data suggested that the *relA* gene and *spoT* gene played pivotal roles in regulating the adaption of the growth of strain 2P24 to nutrient downshift.

3.6. Effect of the *relA* gene and *spoT* gene on stress tolerance

Given the regulatory effect of (p)ppGpp on bacterial physiological processes, the survival rate of the *relA*, *spoT*, and *relA spoT* double mutants were examined under various stress conditions. Our data indicated that no differences in growth curve were found between the *relA*, *spoT*, or *relA spoT* double mutant and the wild-type when *P. fluorescens* cells were grown at pH 7 or at pH 9 (Fig. 7-A and B). However, the inactivation of *relA* and *spoT* significantly impaired the growth of strain 2P24 at pH 5 (Fig. 7-C).

To measure the effect of *relA* and *spoT* genes on high osmotic pressure, the survival of strain 2P24 and its derivatives was determined in the presence of 0.3 mol L^{-1} NaCl. As shown in Fig. 7-D, the *spoT* mutant showed remarkably increased survival rate compared with that of strain 2P24 at 0.3 mol L^{-1} NaCl. Meanwhile, the *relA spoT* double mutant showed decreased viability compared with that of the wild-type at 0.3 mol L^{-1} NaCl.

Next, the susceptibility of strain 2P24 and its derivatives to H_2O_2 was determined using disk diffusion assays. After treatment with a high concentration of H_2O_2 (1 mol L^{-1}), the *spoT* mutant showed markedly increased survival, whereas the *relA spoT* double mutant showed decreased viability compared with that of the wild-type (Fig. 7-E).

To explore the (p)ppGpp-mediated stress tolerance

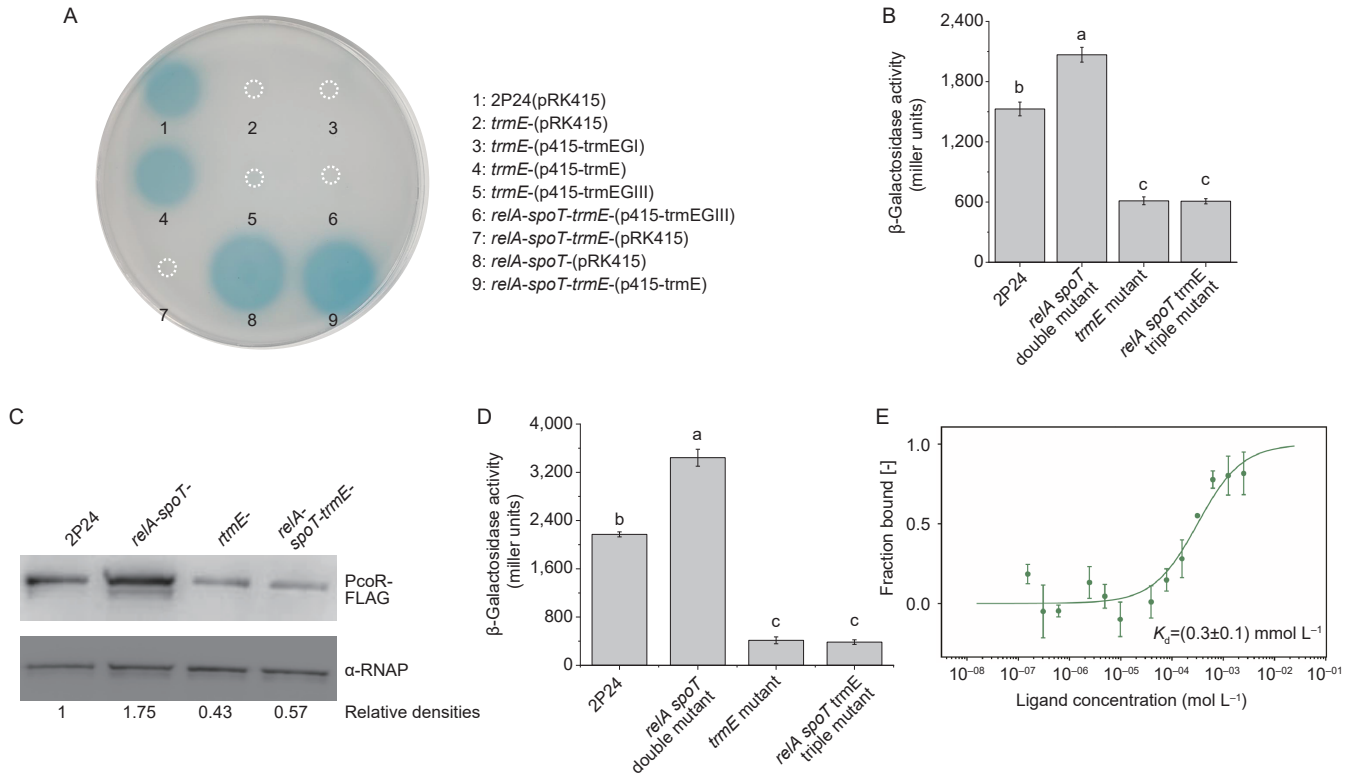


Fig. 5 Regulation of the PcoI/PcoR QS system by (p)ppGpp requires the *trmE* gene. A, production of AHLs of strain 2P24 and its derivatives was determined using the *Agrobacterium tumefaciens* NTL4 (pZLR4) bioreporter. B, β-galactosidase activity of the transcriptional *pcoR-lacZ* was determined in strain 2P24, the *relA spoT* double mutant, the *trmE* mutant, and the *relA spoT trmE* triple mutant. C, Western blot analysis was performed to determine the levels of PcoR in wild-type *Pseudomonas fluorescens* and its derivatives. D, plasmid-borne *pcoI-lacZ* fusion on p970Km-pcoIp was determined in strain 2P24, the *relA spoT* double mutant, the *trmE* mutant, and the *relA spoT trmE* triple mutant. E, MST showing that TrmE forms a complex with (p)ppGppp (K_d, 0.3 mmol L^{-1}). All experiments were performed in triplicate with similar results. Bars indicate one standard deviation from the mean (n=3), while different letters above columns indicate significant differences (P<0.05) according to one-way ANOVA with Tukey's multiple comparisons tests.

mechanism, the expression of the oxidative stress regulator-encoding gene *oxyR* and the catalase-encoding gene *katB* was determined in strain 2P24 and its derivatives. Our data showed that the deletion of the *spoT* gene in *P. fluorescens* 2P24 resulted in the upregulation of *oxyR* and *katB*. Moreover, compared with the wild-type, the promoter activity of *oxyR* and *katB* was impaired in the *relA spoT* double mutant (Fig. 7-F). Collectively, these data suggested that (p)ppGpp regulated stress tolerance by influencing the expression of *oxyR* and *katB*.

3.7. *relA* gene and *spoT* gene influence cell motility

(p)ppGpp directly regulated the expression of flagellar cascade, which is necessary for cell motility (Lemke *et al.* 2009). Therefore, to investigate whether (p)ppGpp influenced the cell motility of strain 2P24, the swimming motility of strain 2P24 and its derivatives was examined on a 0.3% semisolid agarose plates. The results indicated that the swimming motility of the *spoT* mutant was remarkably reduced compared with that of the wild-type (Fig. 8-A). The complementation of the *spoT* gene with p415-spoT restored the swimming motility. Moreover, the mutation of *relA* remarkably increased bacterial swimming motility. The swimming zone formed by the *relA spoT* double mutant was remarkably increased compared with that of the *relA* mutant. Analysis of the swarming motility of 2P24 and its derivatives indicated that the swarming motility was greatly increased in the *relA spoT* double mutant but to a lesser extent than in the *relA* mutant compared with that of the wild-type (Fig. 8-B). However, the mutation of *spoT* remarkably reduced bacterial swarming motility (Fig. 8-B). Collectively, these data suggested that the *relA* gene and the *spoT* gene played a vital role in influencing the motility of *P. fluorescens*.

3.8. (p)ppGpp influenced the biofilm formation and root colonization of *P. fluorescens*

To verify whether (p)ppGpp affected biofilm formation, the biofilm production of 2P24 and its derivatives was measured using the crystal violet assay. As shown in Fig. 9-A, the *spoT* mutant produced less biofilm than the wild-type. Conversely, the biofilm formation was markedly enhanced in the *relA* mutant compared with that of the wild-type. Moreover, the mutation of *relA* and *spoT* had further improved the biofilm formation. These results demonstrated that the biofilm formation was affected by (p)ppGpp in *P. fluorescens*.

Biofilm formation is crucial for rhizobacteria to colonize on the plant roots, which exhibited beneficial effects on plants. To assess whether strain 2P24 and its derivatives differed in

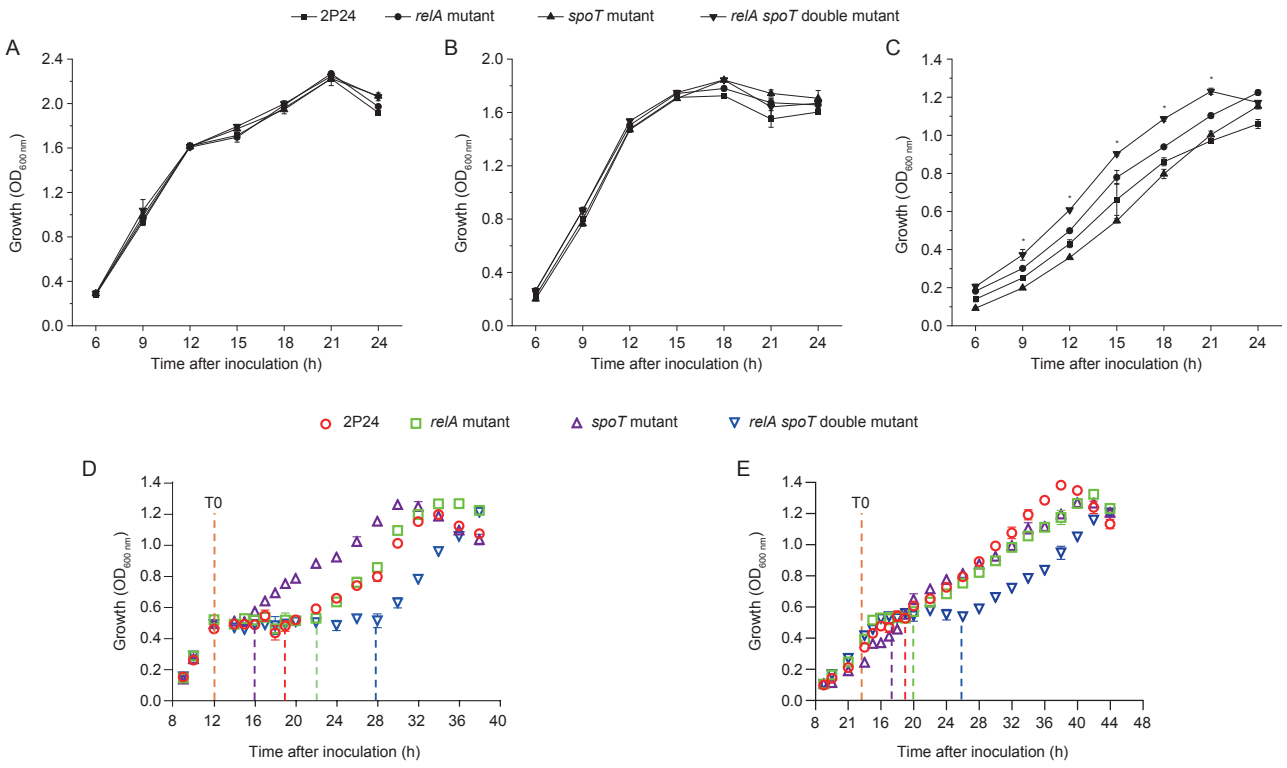


Fig. 6 The effect of *relA* and *spoT* on growth of *Pseudomonas fluorescens*. A–C, growth of strain 2P24, the *relA* mutant, the *spoT* mutant, and the *relA spoT* double mutant was measured in KM liquid medium (A), LB liquid medium (B), and M9 liquid medium (C), respectively. D, growth adaption of *Pseudomonas fluorescens* to amino acid downshift during different (p)ppGpp level. E, growth adaption of *P. fluorescens* to glucose downshift during different (p)ppGpp level. All experiments were performed in triplicate with similar results. Asterisks indicate significant differences (P<0.05) determined using one-way ANOVA with Tukey's multiple comparisons tests in comparison with OD_{600} of the wild type and bars indicate one standard deviation from the mean (n=3).

root colonization, the populations of the wild-type and mutant strains colonized on the wheat roots were monitored under greenhouse conditions. Our data indicated that the *spoT* mutant showed a remarkable reduction in root colonization compared with that of the wild-type at 7 days after inoculation (Fig. 9-B). However, colonization by the *relA spoT* double mutant was markedly higher than that of the wild-type strain. These results indicated that (p)ppGpp was important for the rhizosphere colonization of *P. fluorescens*.

4. Discussion

To adapt to the rhizosphere environment, plant-beneficial *Pseudomonas* spp. must rely on the complicated signaling cascade to respond to environmental cues, such as nutrient deficiency, and the stringent response mediated by (p)ppGpp can coordinate the trade-off between physiological transitions and growth adaption to nutrient starvation (Zhu and Dai 2023). In this study, the involvement of (p)ppGpp in the PcoI/PcoR QS system and root colonization of *P. fluorescens* was investigated. Our data showed that the mutation of the *spoT* gene resulted in the repression of AHL production, whereas the mutation of *spoT* and *relA* resulted in the induction of the production of AHLs, indicating that (p)ppGpp was involved in the regulation of the PcoI/PcoR QS system (Fig. 2). Furthermore, the (p)ppGpp-mediated regulation of the PcoI/PcoR QS system requires a functional TrmE, a tRNA modification GTPase that could directly bind to (p)ppGpp (Fig. 5). In addition, (p)ppGpp was required for motility, biofilm formation, resistance to environmental stress and root colonization of strain 2P24. Therefore, these data suggest that plant-beneficial *Pseudomonas* spp. could utilize (p)ppGpp to sense and respond to fluctuation in environmental nutrient for fine tuning of their biocontrol traits.

A previous study indicated that the complex Gac/Rsm signal cascade played key roles in controlling the PcoI/PcoR QS system (Yan *et al.* 2009). Interestingly, several studies indicated a specific link between the Gac/Rsm signal cascade and (p)ppGpp signaling. In *P. protegens* CHA0, the mutation of the *gacS* gene or the *gacA* gene remarkably enhanced the (p)ppGpp level (Takeuchi *et al.* 2012). Conversely, the expression of *gacS* or *gacA* was greatly decreased in the *relA spoT* double mutant when compared with that of the wild-type. The expression of *rsmY* and *rsmZ* sRNA genes, which are directly regulated by the GacS/GacA system, was markedly reduced in the *relA spoT* double mutant compared with that of the wild-type (Takeuchi *et al.* 2012). However, our data showed that no differences in the expression of *rsmX*, *rsmX1*, *rsmY*, and *rsmZ* were observed between the *relA spoT* double mutant and the wild-type (Fig. 3-A), suggesting that strain 2P24 likely uses a different signaling pathway to execute the biological function of this important stringent signal (p)ppGpp. By contrast, the determination of the relative intracellular (p)ppGpp concentration indicated that no remarkable difference in (p)ppGpp levels was observed

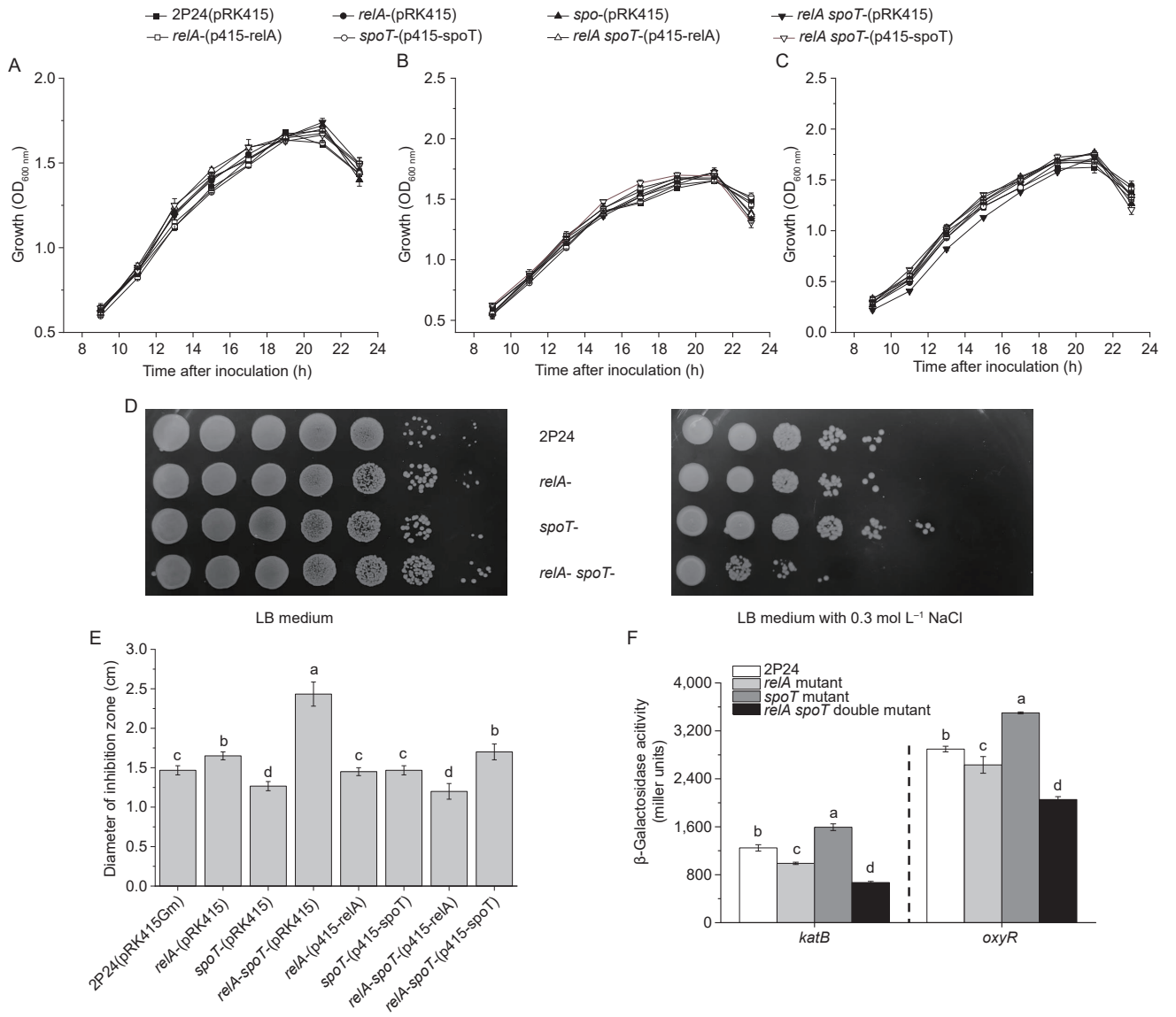


Fig. 7 The effect of the *relA* gene and the *spoT* gene on stress tolerance in *Pseudomonas fluorescens*. A–C, growth of strain 2P24 and its derivatives was measured in LB liquid medium at pH 9 (A), pH 7 (B), and pH 5 (C), respectively. D, spot assays of strain 2P24, the *relA* mutant, the *spoT* mutant, and the *relA spoT* double mutant under 0.3 mol L^{-1} NaCl condition. E, inhibition of 1 mol L^{-1} H_2O_2 for strain 2P24, the *relA* mutant, the *spoT* mutant, and the *relA spoT* double mutant was determined by the disk diffusion assays. F, β-galactosidase activity of transcriptional *katB-lacZ* and *oxyR-lacZ* was determined in strain 2P24 and its derivatives. All experiments were performed in triplicate with similar results. Bars indicate one standard deviation from the mean (n=3), while different letters above columns indicate significant differences (P<0.05) according to according to one-way ANOVA with Tukey's multiple comparisons tests.

between strain 2P24 and its *gacA* mutant (Appendix E). In addition, the disruption of the *relA* gene and *spoT* gene could not affect the expression of the sRNA encoding genes *rsmX*, *rsmX1*, *rsmY*, and *rsmZ* of *P. fluorescens* 2P24, indicted that no cross-talk is found between the (p)ppGpp signaling pathway and the GacS/GacA system and the regulation of the PcoI/PcoR QS system mediated by (p)ppGpp is independent of the function of the GacS/GacA system. These data indicated that different organisms have evolved diverse regulatory mechanisms to coordinate physiological metabolisms through (p)ppGpp.

In fact, we present evidence of the regulatory mechanism linking (p)ppGpp to the PcoI/PcoR QS system *via* TrmE. We found that the mutation of *trmE* decreased the production of AHLs, and the deletion of *trmE* in the *relA spoT* double mutant resulted in a phenotype such as the *trmE* mutant (Fig. 5). These results indicate that the regulation of the PcoI/PcoR QS system by (p)ppGpp required the function of TrmE. TrmE encodes a tRNA modification GTPase and plays an essential role in signal transduction, cell proliferation, and protein synthesis (Scrima *et al.* 2005). TrmE has intrinsic GTPase activity, and it could bind and hydrolyze GTP. Recent studies also showed that TrmE could interact with (p)ppGpp by DRaCALA (Zhang *et al.* 2018a). Notably, our data indicated that (p)ppGpp directly binds to TrmE. Moreover, mutation studies showed that the GxxxxGKS/T motif and DxxG motif of TrmE played an important role in regulating the PcoI/PcoR QS system (Fig. 5).

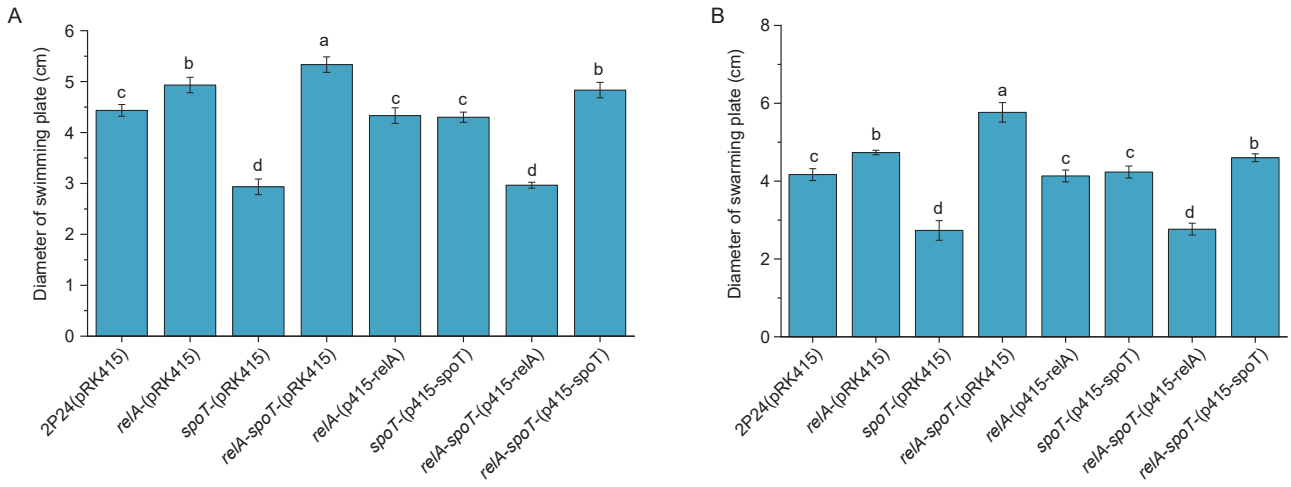


Fig. 8 The effect of the *relA* gene and the *spoT* gene on motility of *Pseudomonas fluorescens*. A, colony diameter of strain 2P24 and its derivatives was measured in swimming assay. B, colony diameter of strain 2P24 and its derivatives was measured in swarming assay. All experiments were performed in triplicate with similar results. Bars indicate one standard deviation from the mean (n=6), while different letters above columns indicate significant differences (P<0.05) according to according to one-way ANOVA with Tukey's multiple comparisons tests.

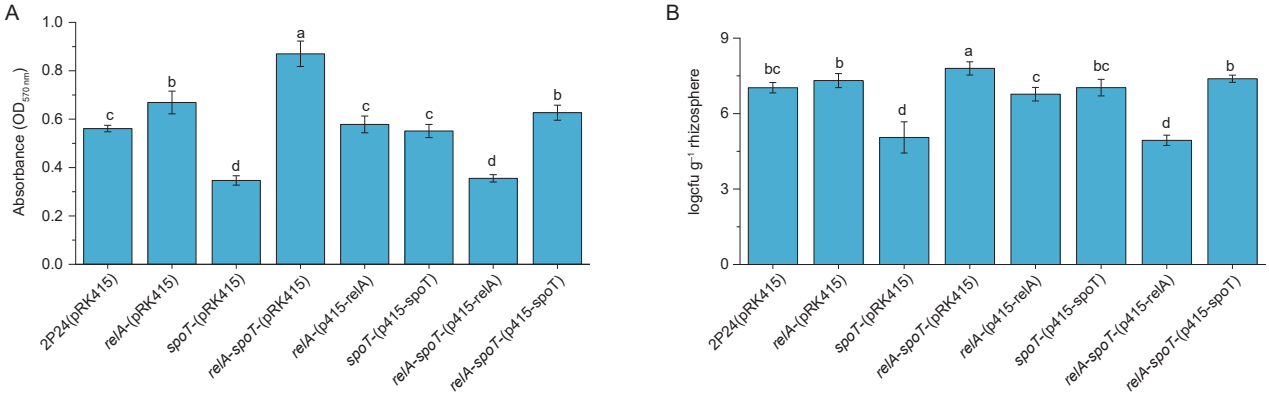


Fig. 9 The *relA* gene and the *spoT* gene influences biofilm formation and root colonization of *Pseudomonas fluorescens*. A, regulation of biofilm formation by the *relA* gene and the *spoT* gene. The biomasses were analyzed for strain 2P24 and its derivatives, and the biofilm was then strained with crystal violet. B, rhizosphere population of strain 2P24 and its derivatives were determined at 14 days after inoculation. All experiments were performed at least three times with similar results. Bars indicate one standard deviation from the mean (n=3), while different letters above columns indicate significant differences (P<0.05) according to a one-way ANOVA with Tukey's multiple comparisons tests.

Interestingly, the GxxxxGKS/T motif or the DxxG motif also played a crucial role in GTP binding (Scrima *et al.* 2005). Combined with previous data, that is, the regulation of 2,4-diacetylphloroglucinol production by TrmE was independent of the Gac/Rsm signaling cascade (Zhang *et al.* 2014), we hypothesized that (p)ppGpp interacts with TrmE and then impedes the function of TrmE of *P. fluorescens* (Fig. 10).

The concentration of (p)ppGpp in cells could be induced by environmental stress, and (p)ppGpp has been observed to mediate bacterial growth in nutrient limitation, stress tolerance, motility, and biofilm formation in many bacteria, including *E. coli*, *P. aeruginosa*, *P. syringae* pv. *tomato*, *Sinorhizobium meliloti*, and *Xanthomonas campestris* pv. *campestris* (Chatnaparat *et al.* 2015; Pacios *et al.* 2020). Our results are consistent with previous reports showing that the *relA* and *spoT* genes play an important role in the adaptation of bacterial growth to amino acid downshift or carbon downshift (Zhu and Dai 2023). Our study also indicated that the mutation of *relA* and *spoT* remarkably impaired the growth of strain 2P24 at pH 5, and increased the sensitivity to H_2O_2. (p)ppGpp influenced the trade-off relation between growth rate and other biological traits in many bacteria (Wang *et al.* 2015). In principle, evolution should impel bacterial species to balance these phenotypes in accordance with their surrounding environment. Therefore, further investigations are necessary to assess whether the (p)ppGpp-binding protein TrmE is involved in regulating the above-mentioned phenotypes and its specific mechanism in *P. fluorescens* 2P24.

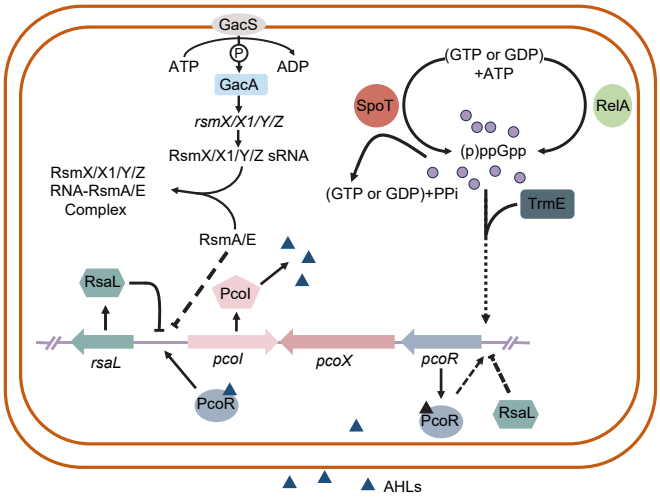


Fig. 10 Working model of the (p)ppGpp signaling pathway on the PcoI/PcoR QS system in *Pseudomonas fluorescens*. In strain 2P24, the PcoI/PcoR QS system was regulated by the Gac/Rsm signaling cascade. In this work, we observed that the regulation of the PcoI/PcoR QS system mediated by (p)ppGpp is independent of the function of the GacS/GacA system. Furthermore, (p)ppGpp-mediated regulation of the PcoI/PcoR QS system requires a functional TrmE, a tRNA modification GTPase that could directly bind to (p)ppGpp.

5. Conclusion

Overall, this work characterized the function of *relA* and *spoT* genes in the PcoI/PcoR QS system, and the (p)ppGpp-binding protein TrmE was necessary for the regulation of the PcoI/PcoR QS system by (p)ppGpp. In addition, (p)ppGpp-mediated stringent response coordinately modulated other biocontrol traits, such as motility, biofilm formation, and rhizosphere colonization. Our data provided new insights that the connection between the PcoI/PcoR QS system and the (p)ppGpp signaling pathway which helping plant-beneficial *Pseudomonas* spp. to achieve a balance between adaptation in rhizosphere and its biocontrol capacity.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscrlpt.

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