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RESEARCH ARTICLE

Negative Regulation of RpoS-mediated STM1703 in Biofilm Formation of *Salmonella* Pullorum

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ABSTRACT

Both alternative sigma factor RpoS and ubiquitous secondary messenger c-di-GMP participate in the biofilm forming of *Salmonella* Pullorum; however, the relationship between RpoS and c-di-GMP-regulated genes during biofilm forming remains unclear. In this study, nine genes related with c-di-GMP regulation were found to be differentially expressed (P<0.01) by RNA-seq analysis when compared with *S.* Pullorum strain S9 and its *rpoS* deletion strain S9S. Specifically, the *rpoS* deletion strain S9S had higher transcription level of gene STM1703 and lower c-di-GMP concentration and biofilm-forming ability than S9 (P<0.01). The STM1703 gene deletion in strains S9 and S9S significantly enhanced the c-di-GMP concentration and the biofilm-forming ability (P<0.01). qRT–PCR analysis showed that *rpoS* deletion or P193L substitution in RpoS increased the transcription level of the STM1703 gene by decreasing the transcription levels of the *csrA* and STM1344 genes (P<0.01). Overall, RpoS-mediated STM1703 negatively regulates the biofilm formation of *S.* Pullorum by degrading c-di-GMP.

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INTRODUCTION

Salmonella can form biofilms to resist antimicrobials (Gonzalez *et al.*, 2018), host defense (MacKenzie *et al.*, 2017) and desiccation (Chen *et al.*, 2021). Biofilm formation linked with environmental stresses involves multiple regulators. The 193 residue of sigma factor RpoS is important for forming biofilms in *S.* Pullorum (Feng *et al.*, 2020). Other sigma factor like RpoE has also been identified to affect biofilm formation of *S.* Pullorum (Huang *et al.*, 2015). Deletion of the response regulator OmpR has been confirmed to reduce the biofilm forming ability in *S.* Pullorum (Lu *et al.*, 2012). Two component system regulated genes like *pagC* or *citB* negatively regulate the biofilm formation of *S.* Pullorum (Kang *et al.*, 2022; Lu *et al.*, 2020).

Molecule 3′-5′-cyclic diguanylic acid (c-di-GMP) also participates in the regulation of biofilm formation as a ubiquitous secondary messenger in bacteria (Ahmad *et al.*, 2017). C-di-GMP regulates the phenotype of bacteria due to change of its concentration. High c-di-GMP concentration in bacterial cells promotes bacteria to form sedentary biofilm phenotype, while low c-di-GMP concentration in bacterial cells favors its motility phenotype (Wang *et al.*, 2018). The c-di-GMP synthesis is controlled by diguanylate cyclases (DGC) while the c-di-GMP degradation is regulated by phosphodiesterases (PDE) (Jenal *et al.*, 2017). Proteins with GGDEF domains possesses DGC activity (Ryjenkov *et al.*, 2005), while proteins involving EAL or HD-GYP domain contribute to PDE activity (Christen *et al.*, 2005; Schmidt *et al.*, 2005). One single protein can contain one or different combinations of these GGDEF or EAL domains.

Bacteria may have multiple proteins involving GGDEF or EAL domains. For example, 5 proteins with GGDEF domain, 7 proteins with GGDEF-EAL domain and 8 proteins with EAL domain have been found in *S.* Typhimurium (Romling, 2005). Proteins STM4551 with GGDEF domain and STM4264 with EAL domain have been confirmed to regulate intracellular c-di-GMP levels and thus regulate the biofilm forming of bacteria (Simm *et al.*, 2007; Solano *et al.*, 2009). Although *S.* Pullorum may share these genes with *S.* Typhimurium, the relationship between RpoS and c-di-GMP-regulated genes in *S.* Pullorum remains unclear. In this study, the transcriptomes of *S.* Pullorum strain S9 and its *rpoS* deletion mutant were determined, the genes related to c-di-GMP regulation were screened and the function of gene STM1703 was evaluated.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions: All the bacterial strains and plasmids of this study are listed in Table 1. Luria-Bertani (LB, Oxoid) broth and 1.5% LB agar medium were prepared for the culture of strains. Antibiotics were mixed with the medium if necessarily at the following concentrations: 20μg/mL chloramphenicol and 100μg/mL ampicillin. Biofilm detection were performed with tryptic soy broth (TSB, BD) diluted 1:10 in distilled water.

RNA-seq analysis: *S.* Pullorum strains S9 and S9S were cultured sedentarily with diluted TSB in small dishes (60mm, Corning) at 28°C for 24 h to form biofilms and S9S was cultured in glass bottles with shaking at 220 rpm to obtain planktonic bacteria. The biofilm state bacteria were collected by scraping from the bottom of the dishes, while the planktonic state bacteria were accumulated by centrifugation. After removal of the medium, samples were flash-frozen in liquid nitrogen for 2 h and sent to the company with dry ice to extract total RNA. Sample quality analyzing, sequencing and analysis services were performed by Personalbio Company in China and each sample contained two replicates.

Quantitative real-time PCR (qRT–PCR) analysis: qRT–PCR analysis was measured as previously described (Feng *et al.*, 2020). Primer pairs for amplification of *gyrB*, STM1703, *csrA*, and STM1344 genes were listed in Table 2.

Construction of mutant and complemented strains: The deletion of STM1703 gene in *S.* Pullorum strains S9 and S9S was constructed by lambda red-recombinant system and verified by PCR amplification (El Hag *et al.*, 2017). The mutants were named S9Δ1703 and S9SΔ1703.

The STM1703 gene was amplified using the primer pair 1703-HF/HR (Table 2) to construct complementary strains. The expression plasmid pGEX-6P-1-1703 was constructed by cloning of the PCR products into pGEX-6P-1 with digestion of the restriction endonucleases *Bam*HI and *Xho*I. The complementary strains S9Δ1703R and S9SΔ1703R were obtained by electroporation of pGEX-6P-1-1703 into *S.* Pullorum S9Δ1703 and S9SΔ1703.

Biofilm assays: Crystal violet staining and quantification tests were performed as previously described (Lu *et al.*, 2012; Stepanovic *et al.*, 2007). Briefly 100 μL of each bacterial suspension was added to 96-well U-bottomed plates (Corning) and incubated at 28°C for 24 h without shaking to form biofilms. The wells were washed three times with distilled water and added with100μL of 0.4% crystal violet for 20 min. After washing the wells, the remaining crystal violet staining was to represent the biofilm forming ability of each strains. Finally, the optical density (OD_{550}) was measured to quantify the biofilm forming ability after solubilizing with 100μL of 25% acetone with anhydrous ethanol.

Two of biofilm components, curli fimbriae and cellulose, were determined by inoculating the bacteria on Congo red plates and LB agar plates containing 200μg/mL calcofluor (Sigma–Aldrich) respectively and incubating at 28°C for 4 days (Anriany *et al.*, 2006).

Extraction of c-di-GMP: The c-di-GMP extraction was carried out as described before (Petrova and Sauer, 2017). C-di-GMP were collected from cell pellets per 15mg in this study.

Quantification of c-di-GMP: The c-di-GMP concentration was detected by Agilent 1260 high-performance liquid chromatography (HPLC) containing a 253nm UV detector. Reverse-phase C18 Agilent column (4.6 × 250mm; 5μm) with a flow rate of 1mL/min was prepared for the separation. The HPLC column was equilibrated and eluted using an isocratic elution solvent, including 75% 10mM ammonium acetate in water and 25% 10mM ammonium acetate in methanol.

To generate c-di-GMP standard curve, serial dilutions of c-di-GMP standard (Sigma–Aldrich) containing 0, 0.625, 1.25, 2.5, 5 and 10pmol/300μL were prepared. The peak height of each standard concentrations was obtained by HPLC analysis software, and the amount of c-di-GMP in pmol vs. the peak height was used to plot a standard curve.

C-di-GMP samples were detected as described before after suspending with 300μL of Nanopure water. Finally, the amounts of c-di-GMP were analyzed through the standard curve and normalized to 1 mg extracts.

Statistical analysis: Graph plotting and statistical analysis were conducted by GraphPad Prism 6. All the data were calculated as mean and standard deviation (Mean ± SD). The statistical analysis was assessed by a two-tailed *t* test and two-way ANOVA. If the *P* values was less than 0.05, then the results were considered significant.

RESULTS

The RNA-seq results of the *S.* Pullorum strain and its *rpoS* deletion mutant during biofilm formation: To explore c-di-GMP related genes in *S.* Pullorum during biofilm forming, the transcriptomes of S9 and its *rpoS* deletion mutant in the planktonic and biofilm states were determined by RNA-seq. More than 25 million effective reads were generated. After checking the base quality of each effective reads and equilibrating the base composition of each sample, the results indicated that the sequencing data could be further analyzed. With the annotated genes provided in the reference genome database (https://www.

Table 1: Bacterial strains and plasmids used in this work

Strains or plasmids	Description	References
Strains		
S. Pullorum		
S9 (S9-PC-wt)	Wild- type strain S11923-3 containing RpoS with residue 193P and 293C	(Feng et al., 2020)
S9S	S11923-3 without *rpoS* gene	(Feng et al., 2020)
S9Δ1703	S11923-3 without STM1703 gene	This work
S9Δ1703R	S11923-3Δ1703 containing pGEX-6P-1-STM1703	This work
S9SΔ1703	S11923-3Δ*rpoS* without STM1703 gene	This work
S9SΔ1703R	S11923-3Δ*rpoS*Δ1703 containing pGEX-6P-1-STM1703	This work
S9-LR	S11923-3 containing RpoS with residue 193L and 293R	(Feng et al., 2020)
S9-PR	S11923-3 containing RpoS with residue 193P and 293R	(Feng et al., 2020)
Plasmids		
pKD46	Lambda red helper plasmid expressing homologous recombinase	(He *et al.*, 2008)
pKD3	Plasmid knockout vector Cm^R	(He *et al.*, 2008)
pCP20	Plasmid knockout vector Amp^R & Cm^R	(He *et al.*, 2008)
pGEX-6P-1	Prokaryotic expression vector Amp^R	Novagen
pGEX-6P-1-1703	pGEX-6P-1 expressing STM1703 from S9	This study

Table 2: Primers used in this study

Primers name	Sequences of primer (5'-3')	Purpose
1703-F	ATGATGAAACAGATTCAGGAACAAACGG	Cloning of 1703
1703-R	TTAACGCATTTTTTTCGTCTGATAACGT	
Q-gyrB-F	ACGCGTCTGTTGACCTTCTTC	Quantitative real-time PCR
Q-gyrB-R	CTGTTCCTGCTTACCTTTCTTCAC	
Q-1703-F	GATGTGGTTCGCCAGATTGC	
Q-1703-R	GCGTGTCGTTTTCGATCAGG	
Q-csrA-F	GAATGCTGATTCTGACTCGTCG	
Q-csrA-R	AGCCTGGATACGCTGGTAGA	
Q-1344-F	TTCAGCAGCGAGCTGAAATG	
Q-1344-R	CCATAGTCCGCCCTGGAATG	
1703-DF	ATGCGAAGAGTACACGGGCTTAAAAGAGCGAGATGTAATGTGTAGGCTGGAGCTGCTTC	Cloning of 1703-*cat*
1703-DR	ATGGATATCTCTGACGAAAGCCTGATCCAGTTTAACGGCCATATGAATATCCTCCTTAG	
1703-HF	CGGGATCCATGATGAAACAGATTCAGGAACAAACGG	Construction of pGEX-6P-1-1703
1703-HR	CCGCTCGAGTTAACGCATTTTTTTCGTCTGATAACGT	

Table 3: Differentially expressed genes related to c-di-GMP regulation during biofilm formation in *S.* Pullorum

Gene ID	Length	Regulation	Description
S9SBF vs. S9SFY			
gene-SEEP9120_RS03935	1064	Up	probable diguanylate cyclase
gene-SEEP9120_RS17355	1220	Down	diguanylate cyclase
gene-SEEP9120_RS11540	1982	Down	cyclic di-GMP phosphodiesterase STM1703
gene-SEEP9120_RS09785	713	Down	hypothetical protein YdiV (STM1344)
gene-SEEP9120_RS17985	185	Up	carbon storage regulator (CsrA)
S9SBF vs. S9BF			
gene-SEEP9120_RS11540	1982	Down	cyclic di-GMP phosphodiesterase STM1703
gene-SEEP9120_RS09785	713	Down	hypothetical protein YdiV (STM1344)
gene-SEEP9120_RS12545	1130	Down	diguanylate cyclase
gene-SEEP9120_RS07850	2990	Down	probable diguanylate cyclase
gene-SEEP9120_RS09440	1493	Down	GGDEF domain-containing protein
gene-SEEP9120_RS12155	1601	Down	putative cyclic-di-GMP phosphodiesterase AdrB
gene-SEEP9120_RS17985	185	Down	carbon storage regulator (CsrA)

ncbi.nlm.nih.gov/genome/152?genome_assembly_id=246259), differentially expressed genes (DEGs) ($P<0.01$) were quantified and compared between strains S9 and S9S in the biofilm state or between strain S9S in the planktonic state and the biofilm state. After analyzing the DEGs of these two comparison groups, we found remarkable differences in transcriptional responses due to the different colors (Fig. 1A). In addition, results of hierarchical clustering analysis showed that repeat samples were clustered well due to the similarity levels in their gene expression pattern.

A total of 1,537 DEGs were discovered in S9S in the biofilm state when compared with S9S in the planktonic state (Fig. 1B), among which 781 genes were upregulated while 756 genes were downregulated. A total of 1,667 DEGs were discovered in S9S in the biofilm state when compared with S9 in the biofilm state, and were comprised of 752 upregulated DEGs and 915 downregulated DEGs (Fig. 1B). A total of 821 identical DEGs were found in both paired groups (Fig. 1C).

To further analyze the relationship between RpoS and c-di-GMP-regulated genes in *S.* Pullorum, nine DEGs related to c-di-GMP regulation were found (Table 3), including probable diguanylate cyclase genes (1064 bp, 2990 bp), diguanylate cyclase genes (1220 bp, 1130 bp), cyclic di-GMP phosphodiesterase STM1703 gene (1982 bp), hypothetical protein YdiV gene (STM1344, 713 bp), GGDEF domain-containing protein gene (1493 bp), putative cyclic-di-GMP phosphodiesterase AdrB gene (1601 bp), and carbon storage regulator (CsrA, 185 bp). Since c-di-GMP phosphodiesterase STM1703 gene downregulation was found in both paired groups, the STM1703 gene was selected as one of the c – di – GMP regulated genes for further study. qRT–PCR confirmed that gene STM1703 transcript level was decreased in S9S under biofilm conditions compared with S9S under planktonic conditions ($P<0.01$), which was conformed to the results of RNA-seq (Fig. 1D), while the transcript levels of the STM1703 gene increased in S9S compared with those of

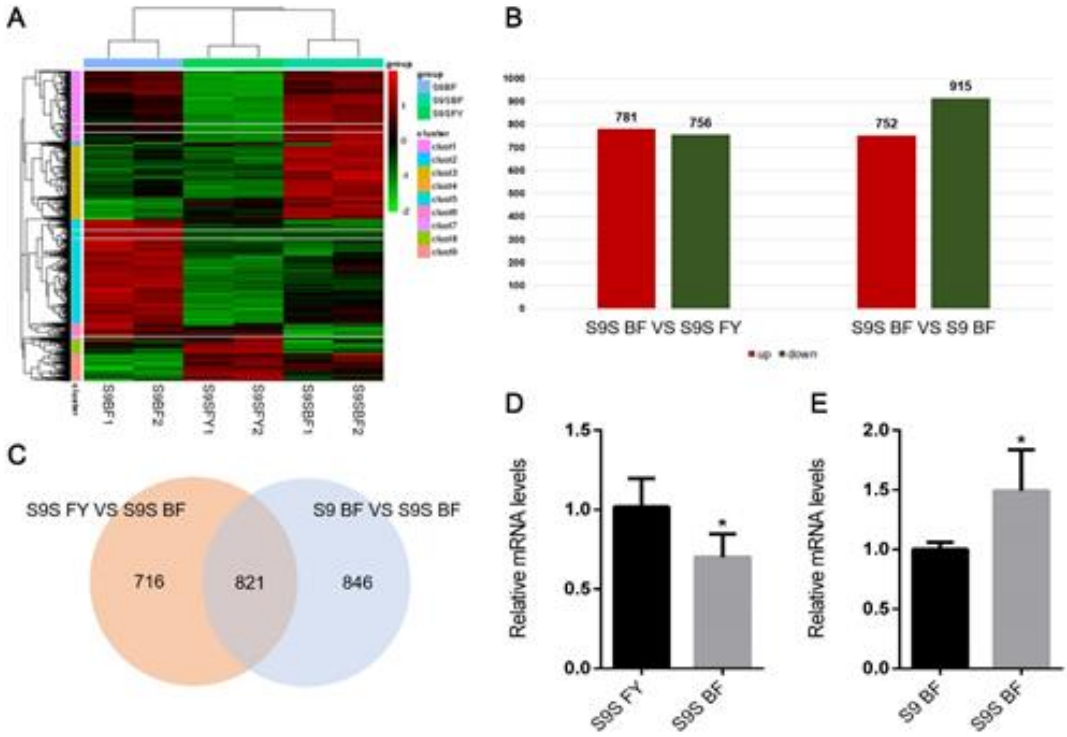

Fig. 1: The RNA-seq results of *S.* Pullorum S9 and S9S during biofilm formation. (A) Heatmap of the differentially expressed genes from the S9SBF (S9S under biofilm state) vs. S9SFY (S9S under planktonic state) paired group, S9SBF vs. S9BF (S9 under biofilm state) paired group. (B) The number of DEGs analyzed in group S9SBF vs. S9SFY and group S9SBF vs. S9BF. (C) The common genes identified from the S9SBF vs. S9SFY paired group and the S9SBF vs. S9BF paired group. (D-E) The mRNA level of the STM1703 gene quantified by qRT–PCR which was normalized to that of the *gyrB* gene. Means and standard deviations were calculated by three independent assays. *P* values less than 0.05 were considered significant.

S9 under biofilm conditions (P<0.01), which was the opposite of the RNA-seq results (Fig. 1E).

STM1703 negatively regulated biofilm forming by reducing c-di-GMP in *S.* Pullorum: To investigate the impacts of STM1703 on biofilm forming in *S.* Pullorum, STM1703 gene deletion mutant S9Δ1703, STM1703 and *ropS* gene double deletion mutant S9SΔ1703 and their STM1703 complementary strains S9Δ1703R and S9SΔ1703R were constructed. After staining by crystal violet, the wild-type strain S9 accumulated the crystal violet as a spot in the plate wells, surrounding the wall and bottom. S9Δ1703 formed a similar staining morphotype as S9, while almost no staining was formed by S9Δ1703R. The *ropS* gene deletion mutant S9S formed circular staining instead of spot staining, and S9SΔ1703 obtained deeper staining but became weaker after complementation with STM1703 (Fig. 2A). The quantification of biofilm forming ability showed that S9Δ1703 and S9 had similar OD_{550} value (P>0.05), while the complementary strain S9Δ1703R had lower OD_{550} value than S9 (P<0.01). Besides, S9S had lower OD_{550} value than S9 (P<0.01) and S9SΔ1703 had higher OD_{550} value than S9S (P<0.01). The OD_{550} value of the STM1703 complementary strain S9SΔ1703R was like that of S9S (P>0.05, Fig. 2B), indicating that STM1703 negatively regulates biofilm formation.

The wild-type strain S9 produced a red, dry, and rough colony (rdar) on the Congo red plates, and the morphology of S9Δ1703 was a deeper and rougher red than that of S9. S9S produced a light-colored and smooth colony, and the morphology of S9SΔ1703 was a deeper red than that of S9S. The morphologies of S9Δ1703 and S9SΔ1703 were restored when STM1703 was complemented. In calcofluor staining assays, the *ropS* gene deletion mutants S9S exhibited reduced fluorescence when compared with the wild-type strain S9, while the STM1703 deletion mutants S9Δ1703 and S9SΔ1703 did not significantly affect the fluorescence (Fig. 2C). This indicates that STM1703 has negative regulation of biofilm forming by reducing the curli production.

To verify the function of STM1703, HPLC detection was performed to quantify the c-di-GMP concentration. As shown in Fig. 2D, there was no significant difference in the c-di-GMP concentration between strains S9 and S9Δ1703 at 8 h or 24 h (P>0.05). S9S had significantly lower c-di-GMP concentration than S9 at 8 h and 24 h (P<0.01), while S9SΔ1703 had significantly higher c-di-GMP concentration than S9S at 8 h (P<0.01), indicating that deletion of the *rpoS* gene significantly reduces the c-di-GMP concentration, and the gene STM1703 deletion significantly increases the c-di-GMP concentration under *rpoS* gene deletion conditions.

RpoS mediates STM1703 to regulate the biofilm formation of *S.* Pullorum: In a previous study, we found that *rpoS* deletion and RpoS P193L reduced biofilm formation in *S.* Pullorum. After quantification of the biofilm-forming ability, the *rpoS* gene deletion mutant S9S had significantly lower OD_{550} value than the wild-type strain S9-193P-293C (S9-PC-wt, P<0.01), and the OD_{550} value of S9-LR with residues P193L and C293R mutation on RpoS was significantly lower than that of S9-PR with residue C293R mutant on RpoS (P<0.01, Fig. 3A). In

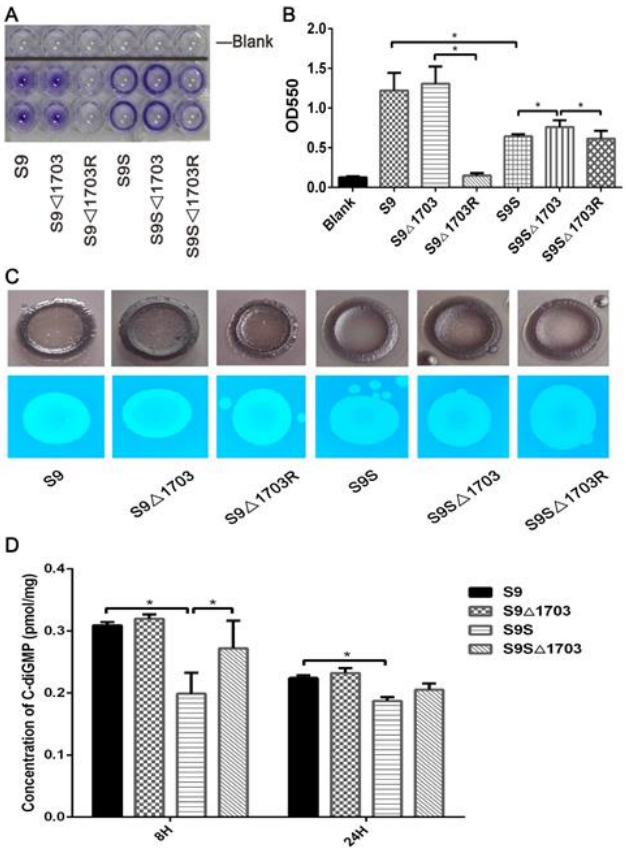

Fig. 2: STM1703 negatively regulated biofilm forming by reducing the c-di-GMP concentration in *S.* Pullorum. (A) Bacteria were grown in 96-well plates and stained by crystal violet. (B) The optical density (OD_{550}) value of crystal violet staining quantification. Experiment results are shown with means and standard deviations. (C) Observation of colony morphology grew on Congo red and calcofluor plates. (D) The c-di-GMP concentration of different strains according to the c-di-GMP standard curve line is shown at 8 h and 24 h. Means and standard deviations were calculated by three independent assays. All experiments were based on S9 and its mutants and were conducted for three times. *P* values less than 0.05 were considered significant.

addition, the OD_{550} value of S9-PR with the residue C293R mutant on RpoS was similar to that of S9-PC-wt ($P>0.05$). To evaluate the effect of RpoS on STM1703 expression, the transcription level of the STM1703 gene after the residue mutation on RpoS was measured by qRT–PCR. S9S had significantly higher transcription level of gene STM1703 when compared with S9 ($P<0.01$). Gene STM1703 transcription level increased significantly in S9-LR during biofilm formation at 8 h and 24 h when compared with that in S9-PR ($P<0.01$). The transcription level of the STM1703 gene decreased significantly in S9-PR during biofilm formation at 8 h and 24 h when compared with that in S9-PC-wt ($P<0.01$, Fig. 3B). To confirm the relationship between c-di-GMP concentration and RpoS regulation, the c-di-GMP concentration was determined by HPLC. Consistent with STM1703 expression, S9S had significantly lower c-di-GMP concentration compared with strain S9-PC-wt ($P<0.01$), and S9-LR also had significantly lower c-di-GMP concentration compared with strain S9-PR ($P<0.01$). In addition, both strains S9-PR and S9-PC-wt had similar c-di-GMP concentration ($P>0.05$, Fig. 3C). These results indicated that deletion of RopS increased the expression of STM1703, reduced the c-di-GMP production and finally reduced biofilm forming. The L193P mutant on RpoS decreased the expression of STM1703, further to increase the c-di-GMP production and biofilm forming in *S.* Pullorum.

To explore the possible regulatory pathway of RpoS on STM1703, the transcription levels of the *csrA* and STM1344 genes under the control of RpoS was determined by qRT–PCR. The transcription levels of *csrA* and STM1344 genes in S9S significantly decreased at 8 h and 24 h when compared with the wild-type strain S9-PC-wt ($P<0.01$), except for *csrA* expression at 24 h ($P>0.05$). The transcription levels of *csrA* and STM1344 genes in S9-LR significantly decreased at 8 h and 24 h when compared with S9-PR levels, while the transcription levels of *csrA* and STM1344 genes in S9-PR significantly increased at 8 h and 24 h when compared with S9-PC-wt levels ($P<0.01$, Fig. 4A-B), indicating that deletion of *rpoS* or residue P193L on RpoS resulted in decreased expression of *csrA* and STM1344 genes.

DISCUSSION

The epidemiological investigation of *S.* pullorum showed that more than sixty-five percentage strains of *S.* Pullorum can form biofilms (Gong *et al.*, 2013). Several regulators have been clarified to regulate biofilm forming ability in *S.* Pullorum (El Hag *et al.*, 2017; Kang *et al.*, 2022; Lu *et al.*, 2020). C-di-GMP possesses many important functions in bacteria, including regulating the rdar biofilm formation and inhibiting the motility and virulence properties (Valentini and Filloux, 2019). C-di-GMP is synthesized or degraded by the corresponding specific enzyme DGCs or PDEs which contain GGDEF and/or EAL domains (Romling *et al.*, 2013). Although more than 20 proteins containing GGDEF and/or EAL domain were discovered in *Salmonella* (Romling, 2005), few studies focused on the relation between RpoS and c-di-GMP regulated genes. In our study, an RNA-seq comparative analysis was performed between *S.* Pullorum strains S9 and S9S during biofilm formation and among S9S strains in the planktonic state and biofilm state. Nine differentially expressed genes were discovered to be related to c-di-GMP regulation, and the mRNA levels of all the screened genes were conformed to the results of RNA-seq, with the exceptions of the probable diguanylate cyclase gene (1064bp) in the S9SBF vs. S9SFY paired group (Data not shown) and the STM1703 gene in the S9SBF vs. S9BF paired group. In addition, the STM1703 and STM1344 genes were identical DEGs in both paired groups, indicating that they may regulate c-di-GMP independently of RpoS in the S9SBF vs. S9SFY paired group and in concert with RpoS in the S9SBF vs. S9BF paired group during biofilm formation.

STM1703 mainly displays phosphodiesterase activity and strongly affects biofilm formation in *S.* Typhimurium (Simm *et al.*, 2007). STM1344 has no effects on the c-di-GMP synthesis or degradation but is involved in rdar morphotype formation and CsgD expression (Romling, 2005). The transcription tendency of STM1703 in qRT-PCR results was not completely consistent with that of RNA-seq, and there was a five amino acid difference in the sequences of *S.* Pullorum and *S.* Typhimurium. So, we focused on the regulatory function of STM1703 in *S.* Pullorum. Biofilm assays proved that STM1703 negatively

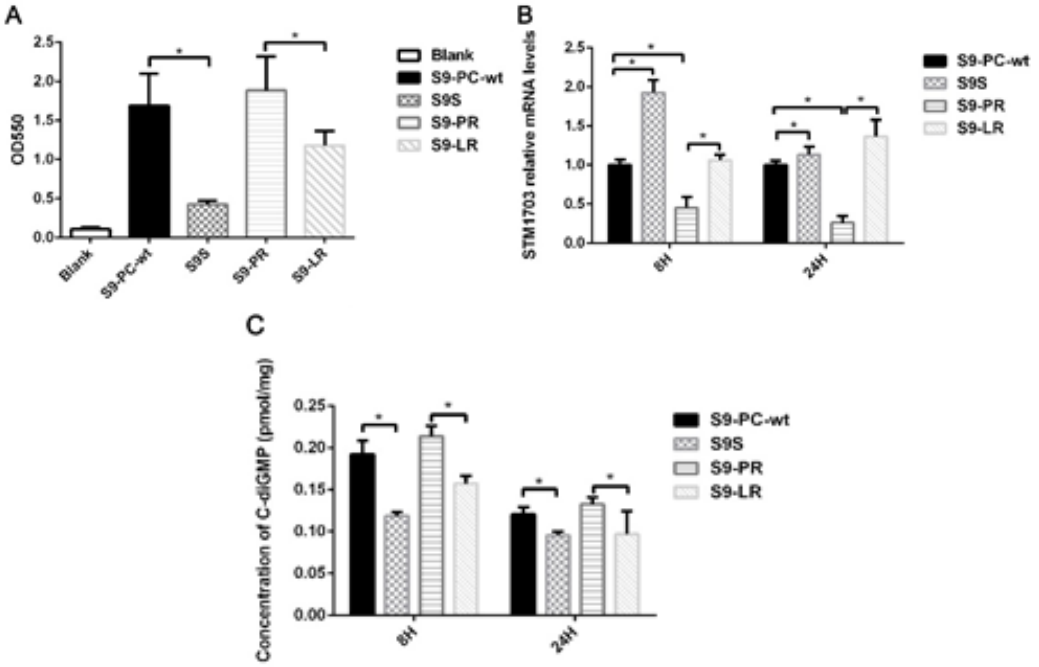

Fig. 3: RpoS mediates STM1703 to regulate the biofilm formation of *S*. Pullorum. (A) The optical density (OD_{550}) value for detection of crystal violet staining. (B) The mRNA level of the STM1703 gene which was normalized to that of the *gyrB* gene. (C) The c-di-GMP concentration in different strains according to the standard curve line is shown at 8 h and 24 h. All of the bars represent the results with means and standard deviations of three repeat assays. The wt indicates the wild-type strain, and PC, PR and LR indicate different amino acid combinations of RpoS substitutions at residues 193 and 293. *P* values less than 0.05 were considered significant.

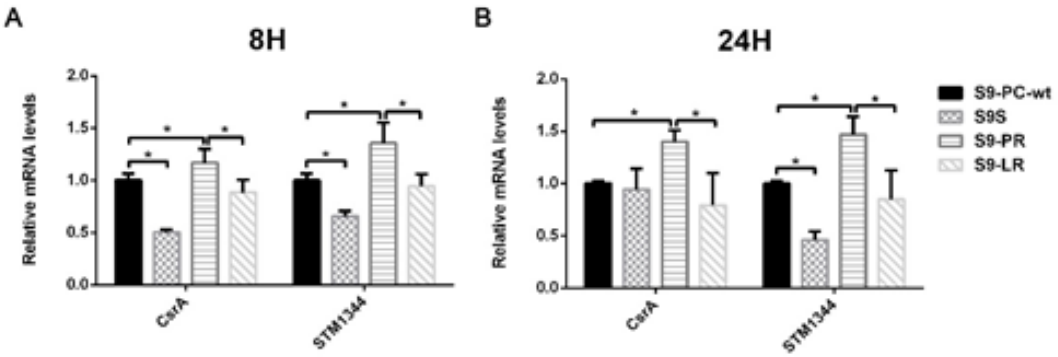

Fig. 4: RpoS regulates STM1703 through CsrA and STM1344. The mRNA levels of *csrA* and STM1344 were quantified by qRT–PCR at 8 h (A) and 24 h (B). Strains were cultured in biofilm state for 8 h and 24 h. Total RNA was extracted, and cDNA was synthesized. The mRNA levels of CsrA and STM1344 were normalized to that of the *gyrB* gene. Means and standard deviations were calculated by three independent assays. The wt indicates the wild-type strain, and PC, PR and LR indicate different amino acid combinations of RpoS substitutions at residues 193 and 293. *P* values less than 0.05 were considered significant.

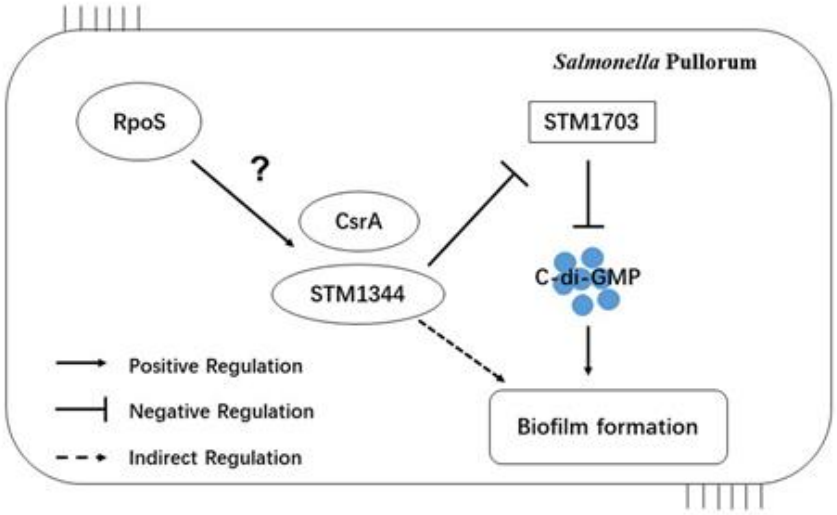

Fig. 5: Schematic diagram demonstrating the interplay among RpoS, STM1703 and c-di-GMP in *S*. Pullorum. STM1703 reduces the biofilm formation of *S*. Pullorum by decreasing the c-di-GMP concentration. RpoS can regulate STM1703 further to affect the biofilm formation of *S*. Pullorm through CsrA and STM1344, although, the specific mechanism remains to be studied. Black arrows represent the positive regulation, whereas flat-headed black arrows represent negative regulation. Direct regulation was symbolized by a solid line, whereas indirect regulation was indicated by a dashed line.

regulated biofilm formation in *S.* Pullorum and that deletion of STM1703 mainly increased curli fimbriae production. HPLC assays confirmed that STM1703 had phosphodiesterase activity, which could degrade c-di-GMP during biofilm forming. All these results indicated that STM1703 had a negative regulation role of biofilm forming by degrading c-di-GMP in *S.* Pullorum, which was consistent with the result in *S.* Typhimurium.

It is worth noting that the negative regulation of STM1703 could function independently of RpoS, as S9SΔ1703 had higher OD_{550} value than S9S and its OD_{550} value was restored after complementation with STM1703. In addition, the transcription level of the STM1703 gene was increased significantly after *rpoS* deletion in S9, indicating an RpoS-dependent response. In fact, regulation of RpoS on YdaM (GGDEF only) and EAL YciR (GGDEF + EAL) genes has been confirmed in *E.* coli (Weber *et al.*, 2005). YciR is the STM1703 homolog in *E.* coli and exhibits RpoS dependence under multiple stressors (Weber *et al.*, 2006). In *S.* Typhimurium, RpoS can directly or indirectly activate the diguanylate cyclase AdrA or *basABZC-bcsEFG* operons further to produce c-di-GMP (Simm *et al.*, 2004). This study found that both deletion of *rpoS* and RpoS P193L substitution increased the transcription level of the STM1703 gene significantly and decreased the concentration of c-di-GMP in *S.* Pullorum, and that the decrease of c-di-GMP concentration was consistent with decreased biofilm formation, indicating that RpoS mediated STM1703 negatively to regulate biofilm formation. It is interesting that RpoS C293R substitution significantly decreased the transcription of STM1703 but did not influence the c-di-GMP concentration, indicating that residue 293 substitution of RpoS may activate other regulators and collectively regulate c-di-GMP release.

Over seven proteins encoding GGDEF and/or EAL domain have been reported to be regulated by CsrA in *E.* coli. (Jonas *et al.*, 2008)., The unconventional proteins with GGDEF and/or EAL domain, STM1827 and STM1703, were downregulated by CsrA directly or indirectly in *S.* Typhimurium (Jonas *et al.*, 2010). STM1344 also integrates into the c-di-GMP network by controlling STM1703, and the deletion of STM1344 enhances the expression of STM1703 (Simm *et al.*, 2009). This study proved that *rpoS* deletion or RpoS P193L substitution decreased the transcription levels of *csrA* and STM1344. In addition, RNA-seq analysis determined that *csrA* and STM1344 genes were significantly downregulated between the S9SBF vs. S9BF paired group (Table 3). Taken together, we speculate that RpoS may upregulate the expression of *csrA* and STM1344 genes, downregulate the expression of STM1703 gene, upregulate the c-di-GMP production, and ultimately increase the biofilm forming ability of *S.* Pullorum (Fig. 5). The residue L193P mutant on RpoS showed a similar regulation pattern.

Conclusion: STM1703 has negative regulation on the biofilm forming of *S.* Pullorum by decreasing the c-di-GMP concentration, and RpoS could mediate the transcription level of the STM1703 gene via the *csrA* and STM1344 genes. This study provides new information for the regulation of RpoS-mediated STM173 during biofilm formation in *S.* Pullorum.

Conflicts of interest: The authors declare that there are no conflicts of interest.

Authors contribution: FZ and PDX contributed to the design. FZ, WN and HM contributed to the research performances. FZ, QT, and PDX contributed to the data analysis. FZ, QT, CSJ, and PDX contributed to the paper composition.

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