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Polyphosphate Kinase Affects Oxidative Stress Response by Modulating cAMP Receptor Protein and *rpoS* Expression in *Salmonella* Typhimurium

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Polyphosphate (polyP) plays diverse physiological functions in prokaryotes and eukaryotes, but most of their detailed mechanisms are still obscure. Here, we show that deletion of polyphosphate kinase (PPK), the principal enzyme responsible for synthesis of polyP, resulted in augmented expression of cAMP receptor protein (CRP) and *rpoS* and lowered H_2O_2 sensitivity in *Salmonella* Typhimurium ATCC14028. The binding of cAMP–CRP complex to *rpoS* promoter and further stimulation of its transcription were proved through electrophoretic mobility shift assay, *lacZ* fusion, and exogenous cAMP addition, respectively. The *rpoS* expression increased in *cpdA* (cAMP phosphodiesterase coding gene) mutant, further suggesting that cAMP–CRP upregulated *rpoS* expression. These results demonstrate that PPK affects oxidative stress response by modulating *crp* and *rpoS* expression in *S.* Typhimurium.

Keywords: cAMP–CRP, *crp*, *ppk*, *rpoS*, *Salmonella* Typhimurium

Inorganic polyphosphate (polyP) is a linear, unbranched polymer of tens to hundreds of orthophosphate residues linked by high-energy phosphoanhydride bonds, and exists in a broad range of organisms from three kingdoms of life. Research in recent years indicates that this ubiquitous biopolymer plays versatile and vital roles in response regulation and metabolism in prokaryotes and eukaryotes. In bacteria, polyP is synthesized by the polyphosphate kinase (PPK), an inner membrane-associated enzyme, and hydrolyzed by exopolyphophatase (PPX) [1, 2]. Bacterial polyP is involved in many important physiological processes, such as fidelity of DNA replication [25, 35], activation of Lon protease [19, 30], stress resistance [36], biofilm formation [32], and virulence [16, 26]. However, most of the underlying mechanisms of its functions remain unclear, including the effect of *ppk* on the *rpoS* expression in *Escherichia coli* and salmonellae.

RpoS has been recognized as an alternative sigma factor (σ^S) of RNA polymerase, which plays roles in general stress response and modulation of stationary phase. In *E. coli*, RpoS controls a regulon of more than 60 genes for stress response and transition from exponential phase to stationary phase [10]. Besides the similar roles as in *E. coli* [13], RpoS is also involved in pathogenesis in *Salmonella* Typhimurium [6, 8, 29]. The regulation of intracellular RpoS is achieved in transcription, translation, protein turnover, and protein activity, among which the transcription regulation is still under relatively insufficient characterization [10]. Striking augmentation of *rpoS* transcription has been observed during growth in rich medium [22], and several effectors, such as cAMP–CRP [40], Fis [11], ArcA [27], ppGpp [12], and polyP [26, 34], have been indentified. Previous research indicates that *ppk* can affect *rpoS* at the transcriptional level with unknown mechanisms [26, 34]. In this work, we attempted to disclose the underlying mechanism of transcriptional regulation of *rpoS* by *ppk*.

Materials and Methods

Bacterial Strains, Media, and Growth Conditions

Bacterial strains used in this study are listed in Table 1. Buffered Luria–Bertani (LB) broth (pH 7.0) contained 1% tryptone (Oxiod), 0.5% yeast extract (Oxiod), 1% NaCl, and 100 mM MOPS (Sigma). cAMP (Sigma) was added at a final concentration of 10 mM. When necessary, media were supplemented with antibiotics at the following concentrations: ampicillin (Ap), 100 μg/ml; kanamycin (Kan), 50 μg/ml.

Chromosomal Deletion of *ppk* and *cpdA*

Deletion of *ppk* and *cpdA* was performed as previously described [7]. Briefly, the phage λ Red recombination system was used to replace *crp* with the *crp*::Kan PCR fragment. Kanamycin cassettes

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Table 1. Strains and plasmids used in this study.

Strain and plasmid	Description	Reference or source
Salmonella Typhimurium strains		
ATCC14028	Wild type	American Type Culture Collection
ST140281	ATCC14028 Δ*ppk*::Kan	This work
ST140282	ATCC14028 Δ*cpdA*::Kan	This work
E. coli strains		
DH5α	Δ*lacU169 recA1 endA1 gyrA96 relA1 hsdR17 thi-1 supE44*	Laboratory stocks
BL21(DE3)	F^-, *ompT*, $hsdS_B(r_B^- m_B^-)$, *dcm*, *gal* (DE3)	Novagen
Plasmids		
pKD46	pINT-ts derivative containing *araC*-P_{araB} and γ β exo DNA fragments	[7]
pKD4	pANTS γ derivative containing an FRT-flanked Kan^R gene	[7]
pFZY1	*galK'–lacZYA* transcriptional fusion vector, Ap^R	[18]
PFrpoS	pFZY1 derivative containing *rpoS* promoter upstream of *galK'–lacZYA*	This work
pFrpoS01	pFrpoS derivative containing mutation in CRP-binding site I	This work
pFrpoS02	pFrpoS derivative containing mutation in CRP-binding site II	This work
pFrpoS03	pFrpoS derivative containing mutation in CRP-binding sites I and II	This work
pFcrp	pFZY1 derivative containing *crp* promoter upstream of *galK'–lacZYA*	This work
pFcya	pFZY1 derivative containing *cya* promoter	This work
pFkatG	pFZY1 derivative containing *katG* promoter	This work
pFkatE	pFZY1 derivative containing *katE* promoter	This work
pET-28a(+)	Expression vector, Kan^R	Novagen
pGcrp	pET-28a(+) derivate containing CRP-coding sequence	This work

from pKD4 were amplified using Dppk-F1/Dppk-R1 and DcpdA-F1/DcpdA-R1 as primers (Table 2), respectively. The mutants with suspected deletion of *ppk* and *cpdA* were checked by PCR using primers Dppk-F2/Dppk-R2 and DcpdA-F2/DcpdA-R2, respectively. Resulting PCR products were sequenced for final confirmation.

Plasmid Construction

Plasmids used in this study are listed in Table 1. Primers used for amplifying promoters are listed in Table 2. Enzymes were purchased from New England Biolabs unless indicated otherwise. PrimerSTAR HS DNA polymerase (Takara) was used in all PCRs.

A mini-F-derived plasmid pFZY1 was used to construct promoter-fused β-galactosidase report plasmids. To construct pFrpoS, the *rpoS* promoter was amplified using PrpoS-R and PrpoS-F primers. The resulting PCR product was purified, digested, and inserted into the EcoRI site of pFZY1. Other plasmids derived from pFZY1 were constructed following a similar procedure. Base-substitution in predicted CRP-binding sites in the *rpoS* promoter was performed as previously described [9] with some modification. In detail, to construct pFrpoS01, primer PrpoS01-F was designed to be complementary to the sequence of the *rpoS* promoter in pFrpoS, except that five bases in the predicted CRP-binding sites were substituted (Fig. 4A). PCR was performed using pFrpoS as the template and PrpoS01-F/PrpoS01-R as primers. After purification, the resulting PCR product was digested with DpnI, and then treated with T4 polynucleotide kinase, and finally self-ligated. The ligation product was transformed into DH5α for plasmid amplification. The pFrpoS02 was constructed following a similar procedure using pFrpoS as the template and PrpoS02-F/PrpoS02-R as primers. The pFrpoS03 was constructed using pFrpoS01 as the template and PrpoS02-F/PrpoS02-R as primers. To construct pGcrp, the gene coding CRP in *Salmonella* Typhimurium ATCC14028 was amplified using primers of Gcrp-F/Gcrp-R. The purified PCR product was digested and inserted into pET-28a(+) between NcoI and XhoI sites. All constructed plasmids were sequenced for insertion.

Protein Expression

The pGcrp was transformed into *E. coli* BL21. When cells grew to OD_{600} of 0.4, expression of CRP was induced by adding 1 mM isopropy-β-D-thiogalactoside (IPTG) (Sigma), and then cells were grown for an additional 3 h. His-tagged CRP protein was purified by a HisLink Protein Purfication Resin according to the manufacturer's instructions (Promega) and finally eluted with 0.05 M potassium phosphate−potassium hydroxide buffer containing 0.5 M KCl and 250 mM imidazole. After dialysis twice with 0.05 M potassium phosphate−potassium hydroxide buffer containing 0.5 M KCl, glycerol was added to a final concentration of 10%. The protein solution was stored at −80°C until use.

Electrophoretic Mobility Shift Assay (EMSA)

The DNA fragment of the native *rpoS* promoter with putative CRP-binding box I or II or both was amplified from the genome of *S.* Typhimurium ATCC14028 using primers DIS-PrpoS01-F/DIS-PrpoS01-R, DIS-PrpoS02-F/DIS-PrpoS02-R, or DIS-PrpoS01-F/DIS-PrpoS02-R, respectively. The mutated DNA fragments of the *rpoS* promoter were amplified using pFrpoS01 or pFrpoS02 as templates and

Table 2. Primers used in this study.

Name	Sequence	Discription
Gene deletion		
Dppk-F1[a]	*ATGGGTCAGGAAAAGCTATATATCGAGAAAGAACT*GT- GTAGGCTGGAGCTGCTTC	To amplify Kan cassette to delete *ppk*
Dppk-R1	*TTAGTCTGGTTGCTCGAGTGATTTGATGTAGTCAT*- CATGGGAATTAGCCATGGTCC	To amplify Kan cassette to delete *ppk*
Dppk-F2	TCATTCTCCAGGCGAAGGTGC	To test *ppk*-deleted clone
Dppk-R2	CGTTCAGCAAACAGCGACAGAC	To test *ppk*-deleted clone
DcpdA-F1	*TTGGAAAGCCTGTTAACCCTACCTCTGGCTGGTGAG-* *GCC*GTGTAGGCTGGAGCTGCTTC	To amplify Kan cassette to delete *cpdA*
DcpdA-R1	*TCAGTATCCTTCCGAAGCGGTATCGGGTCGGAAC-* *CGGG*CATGGGAATTAGCCATGGTCC	To amplify Kan cassette to delete *cpdA*
DcpdAF2	CCGACTGGCTACGATTCTGT	To test *cpdA*-deleted clone
DcpdAR2	AATGCGTGATTACCACCCTC	To test *cpdA*-deleted clone
Plasmid construction		
PrpoS-F	CCGGAATTCGAACGATTTCCGCGATCT	To amplify *rpoS* promoter
PrpoS-R	CGCCGGATCCAAGGTGGCTCCTACCCGTGA	To amplify *rpoS* promoter
PrpoS01-F[b]	**CGATC**CCAGGTCTGCACAAAATTC	To mutate CRP-binding site I in *rpoS* promoter
PrpoS01-R	ACTCCTTGCTGTGCTGCATC	To mutate CRP-binding site I in *rpoS* promoter
PrpoS02-F	**T**G**GGA**CGGTTG**TCAC**AGCGCCTGTAACGGCACCA	To mutate CRP-binding site II in *rpoS* promoter
PrpoS02-R	GCAGGCTTGTTGTTTGGCAA	To mutate CRP-binding site II in *rpoS* promoter
Pcrp-F[c]	CCGGAATTCAACTCCAATGTTTCAATTTTCCTG	To amplify *crp* promoter
Pcrp-R	CGCGGATCCGCGCGGTTATCCTCTGTTATA	To amplify *crp* promoter
Pcya-F	CCGGAATTCAGAGTATGTTAGTTTTCCGGTCAC	To amplify *cya* promoter
Pcya-R	CGCGGATCCGACGTATCGCCTGATGTTGCT	To amplify *cya* promoter
PkatG-F	CCGGAATTCCCTCCAGCTCCCTGCCGGGAGCTT	To amplify *katG* promoter
PkatG-R	CCGGAATTCATCTCAGCTCCCTTTTAAAGTGT	To amplify *katG* promoter
PkatE-F	CCGGAATTCTAATGCTTCACTAAAAGAGGCT	To amplify *katE* promoter
PkatE-R	CCGGAATTCTGAACTCGTCTCCTGCTTTC	To amplify *katE* promoter
Gcrp-F	CATGCCATGGTGCTTGGCAAACCGCAAACAGA	To amplify CRP-coding gene
Gcrp-R	CCGCTCGAGACGGGTGCCGTAGACGACGAT	To amplify CRP-coding gene
DIG-labeled		
DIG-PrpoS01-F	AGCCTGAATGTAGGGCAAAC	DIG-labeled primer for *rpoS* promoter fragments wt, wt1, and mt1
DIG-PrpoS01-R	GCAGGCTTGTTGTTTGGCAA	Primer for wt1 and mt1
DIG-PrpoS02-F	GAGGGCTCAGGTGAACAAA	Primer for wt2 and mt2
DIG-PrpoS02-R	CTTACTGCCTGCAATGTCG	DIG-labeled primer for *rpoS* promoter fragments wt, wt2, and mt2

[a]The italic letters indicate sequence annealing to the specific gene.

[b]The bold letters indicate bases substituted to investigate cAMP−CRP binding.

[c]The letters with underline indicate bases for the restriction endonuclease recognition site: *Bam*HI (GGATCC), *Eco*RI (GAATTC), *Nco*I (CCATGG), *Xho*I (CTCGAG).

DIS-PrpoS01-F/DIS-PrpoS01-R or DIS-PrpoS02-F/DIS-PrpoS02-R as primers, respectively. The DIG gel shift kit (Roche) was used to label DNA fragments and detect signals according to the manufacturer's instructions. Binding reaction was conducted by incubating a 20-fmole labeled DNA fragment with various amounts of purified CRP in 20 µl of 1× binding buffer (10 mM Tris-HCl [pH 8.0], 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 50 µg/ml of bovine serum albumin, 100 µM cAMP) at 37°C for 10 min. Then, 5 µl of loading buffer was added and the mixtures were electrophoresed immediately in 4% native polyacrylamide gel in 0.5× TBE (45 mM Tris-borate, 1 mM EDTA; pH 8.0) containing 100 µM cAMP at 4°C.

β-Galactosidase Activity Assays

Overnight cultures in buffered LB were diluted into 50 ml of fresh buffered LB with the OD_{600} of 0.02 and grown at 37°C with shaking at 210 rpm in a 250-ml flask. Aliquots were taken at indicated time points to measure OD_{600} and β-galactosidase activity. The assay procedure was conducted according to Miller and the specific

activity was expressed in Miller units [28]. Experiments were repeated at least three times and the results were consistent.

Measurement of H_2O_2 Sensitivity

Overnight cultures of wild type (WT) and *ppk* mutant were collected, washed, and resuspended in buffered LB to OD_{60} of 5.0. H_2O_2 was added to various final concentrations. After incubation at 25°C for 30 min, cultures were serially diluted in buffered LB and spread on LB agar plates to determine viable cell numbers. Experiments were repeated at least three times and similar results were observed.

Results

ppk Deletion Resulted in Increased Transcription of *rpoS* and *katE* and Decreased Sensitivity to H_2O_2

Transcription of *rpoS* in *S.* Typhimurium ATCC14028 WT and *ppk* mutant (Δ*ppk*) was monitored in buffered LB that was adopted to avoid possible effect of pH change on the *rpoS* expression. For this purpose, a 786-bp DNA fragment, the major promoter of *rpoS* [20, 31, 37], was cloned into pFZY1 to drive β-galactosidase expression. Significant augmentation of *rpoS* transcription in Δ*ppk* was observed compared with WT after cells entered late-exponential growth (OD_{600}~2.0), without difference in growth rate (Fig. 1). Moreover, transcription of *katE*, a *rpoS*-dependent gene, was tested in WT and Δ*ppk*. *katE* encodes a stationary-phase-induced catalase HP II, which affects cell sensitivity to H_2O_2 in the stationary phase [13]. Consistent with the increase of *rpoS* transcription, Δ*ppk* showed increased transcription of *katE* (Fig. 2A). Meanwhile, Δ*ppk* showed lowered sensitivity to H_2O_2 (Fig. 2B).

ppk Mutant Had an Increased *crp* Expression

lacZ Fusion report plasmids were constructed and the expressions of *cya* and *crp* in WT and Δ*ppk* were accessed.

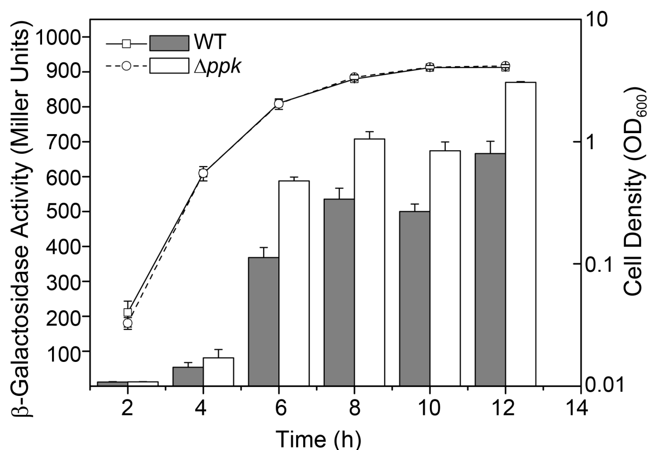

Fig. 1. Effect of the *ppk* deletion on the transcription of *rpoS*.
Cells of WT and Δ*ppk* carrying plasmid pFrpoS were cultured overnight, and subsequently diluted in buffered LB. During growth, aliquots were removed at the indicated time points to measure OD_{600} (lines) and β-galactosidase activity (columns).

Results showed that in the late-exponential phase and stationary phase, Δ*ppk* possessed higher *crp* expression (Fig. 3) but unaffected *cya* expression (data not showed) compared with WT. Given that the cAMP−CRP complex is long considered as one of the regulators modulating *rpoS* transcription in bacteria [21, 22, 39], it raised the question of whether *ppk* affects transcription of *rpoS* through the cAMP−CRP complex.

cAMP−CRP Upregulated *rpoS* Transcription Through Direct Binding to Two Sites Within the *rpoS* Promoter

There are two putative cAMP−CRP binding sites within the *rpoS* promoter (Fig. 4A) in *S.* Typhimurium and *E. coli* [10, 11]. However, no experimental evidence has shown whether cAMP−CRP regulates *rpoS* expression by direct binding to the *rpoS* promoter. To answer this question, we performed a series of electrophoretic mobility shift assays

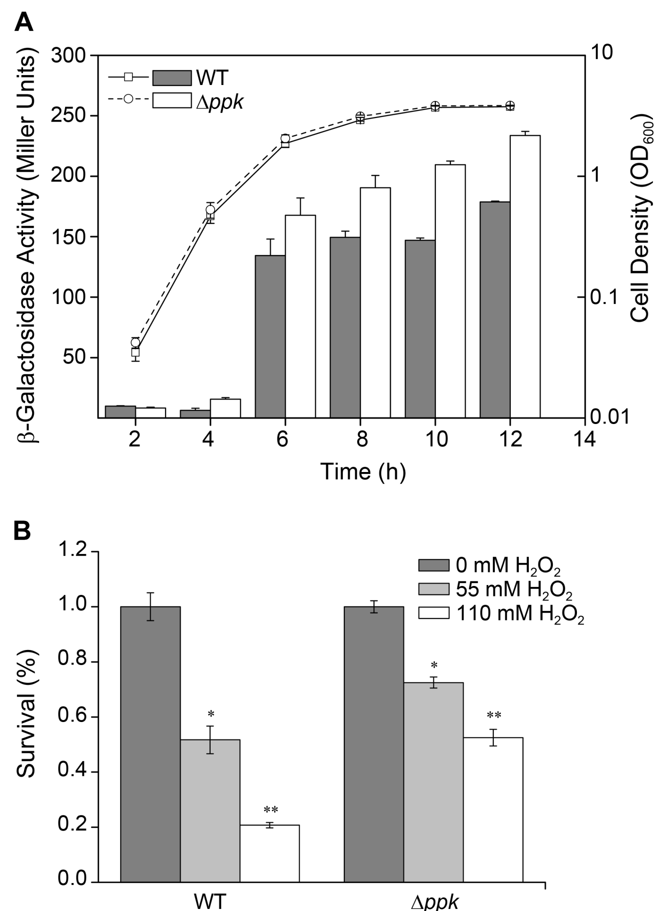

Fig. 2. A. Effect of the *ppk* deletion on the transcription of *katE*.
Transcription of *katE* was measured in WT and Δ*ppk* during growth. Conditions for cell growth and β-galactosidase assay are described in Materials and Methods. **B**. H_2O_2 sensitivity of WT and Δ*ppk*. Cells were exposed to 0 mM, 55 mM, and 110 mM H_20_2. Survival of WT or Δ*ppk* exposed to different concentrations of H_2O_2 is presented as values relative to the number of cells of WT or Δ*ppk* exposed to 0 mM H_2O_2, respectively. Columns with same numbers of asterisk indicate *P* value less than 0.01.

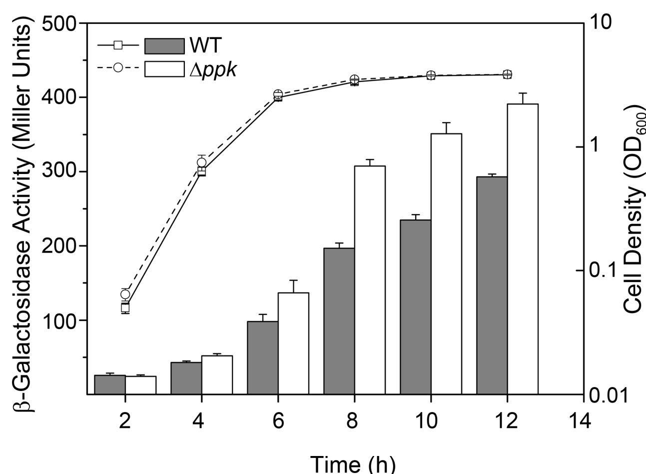

Fig. 3. Transcription of *crp* in *ppk* mutant compared with WT.
Cells of WT and Δ*ppk* carrying plasmid pFcrp were cultured as described in Materials and Methods. At indicated time points during growth, aliquots were collected to measure OD_{600} and β-galactosidase activity.

(EMSA) using native and mutated *rpoS* promoter fragments to determine their interaction with the cAMP−CRP complex. The whole *rpoS* promoter bound to CRP in EMSA (data not shown). Moreover, DIG-labeled promoter fragments with corresponding CRP−binding box I (wt1) and II (wt2) were incubated with various concentrations of CRP from *S.* Typhimurium ATCC14028. As shown in Fig. 4B and 4C, CRP can specifically bind to two fragments in a dose-dependent manner in the presence of cAMP. Meanwhile, DNA fragments with mutations in the two putative binding sites (mt1, wt1 with mutations in CRP box I; mt2, wt2 with mutations in CRP box II) were used in EMSA and no retardance was observed at various CRP concentrations.

In addition, the activity of promoters with mutations in CRP-binding boxes I, or II, and both was analyzed using *lacZ* fusion plasmids. As shown in Fig. 5A, *rpoS* transcription in WT was significantly decreased, driven by promoters with base-substitutions in CRP-binding box I and II, respectively. More decrease was observed when both sites were mutated, further demonstrating the positive role of both sites in *rpoS* transcription.

Furthermore, the effect of exogenous cAMP on the activity of *rpoS* promoter was evaluated. Addition of 10 mM exogenous cAMP stimulated the transcription of *rpoS* driven by the original promoter, and had no effect on the activity of *rpoS* promoter with base-substitutions in both of the CRP binding sites.

rpoS Transcription was Increased in *cpdA* Mutant and the Increase was Abolished When Two cAMP−CRP Binding Boxes in *rpoS* Promoter were Mutated

The *rpoS* transcription was further investigated in the mutant of *cpdA* gene, which encodes a cAMP phosphodiesterase [5, 14, 17]. *cpdA* Deletion resulted in about 30% increase of intracellular cAMP compared with WT (data not shown). As demonstrated in Fig. 6A, there was significant increase in *rpoS* transcription in the *cpdA* mutant (Δ*cpdA*) compared with WT. Besides, the activity of *rpoS* promoter with mutations in both of the cAMP−CRP binding sites was also analyzed and compared in Δ*cpdA*, Δ*ppk,* and WT, respectively. The β-galactosidase activity of overnight cultures indicated that the native *rpoS* promoter possessed higher activities in Δ*ppk* and Δ*cpdA* than in WT, whereas the activity of mutated promoter had no significant difference in all the three strains (Fig. 6B).

DISCUSSION

More and more studies have demonstrated the importance of polyP/PPK in bacterial physiology, a piece of which is on the role of polyP/PPK in stress response. One study reported that the *E. coli rpoS* mutant cannot accumulated polyP in response to nitrogen exhaustion and osmotic stress [3]. Therefore, it is not surprising that polyP/PPK interacts with RpoS, another important regulator of general stress response.

In typical enteric bacteria such as *E. coli* and salmonellae, stress adaptation is mediated by the RpoS protein, the master regulator of general stress response, collectively allowing a tailored transcriptional response to environmental cues [10, 39]. It has been reported that loss of polyP/PPK can affect *rpoS* transcription, but no detailed mechanism was proposed. In our study, an opposite regulation of *ppk* on *rpoS* transcription was observed in *S.* Typhimurium, which is in contrast to previous studies. Reduced *rpoS*–*lacZ* transcription has been reported when overexpressing a yeast potent PPX but maintaining a functional PPK in the *E. coli* cell [34]. The controversy may suggest the participation of both polyP and PPK in the regulation of *rpoS* transcription. Moreover, the differential regulation of *ppk* to *rpoS* transcription should be considered under different growth conditions. For example, McMeechan *et al*. [26] reported a decreased *rpoS* transcription in *S.* Typhimurium F98. Our study indicated that the *ppk* mutant had a significantly higher transcription level of *rpoS* compared with WT in buffered LB medium in the late-exponential phase and stationary phase (Fig. 1). Besides using a *lacZ*-fused, low-copy (one to two copies per cell) report plasmid, real-time PCR was also conducted in our study, which validated that there was a higher mRNA level of *rpoS* in the *ppk* mutant (data not shown). RpoS has been reported to be involved in H_2O_2 resistance. There are two kinds of hydroperoxidases (HP) in cells, HP I and HP II, which are encoded by *katG* and *katE*, respectively. As to *katG*, there is still debate on whether *rpoS* regulates its expression [15, 38]. More experimental evidence has

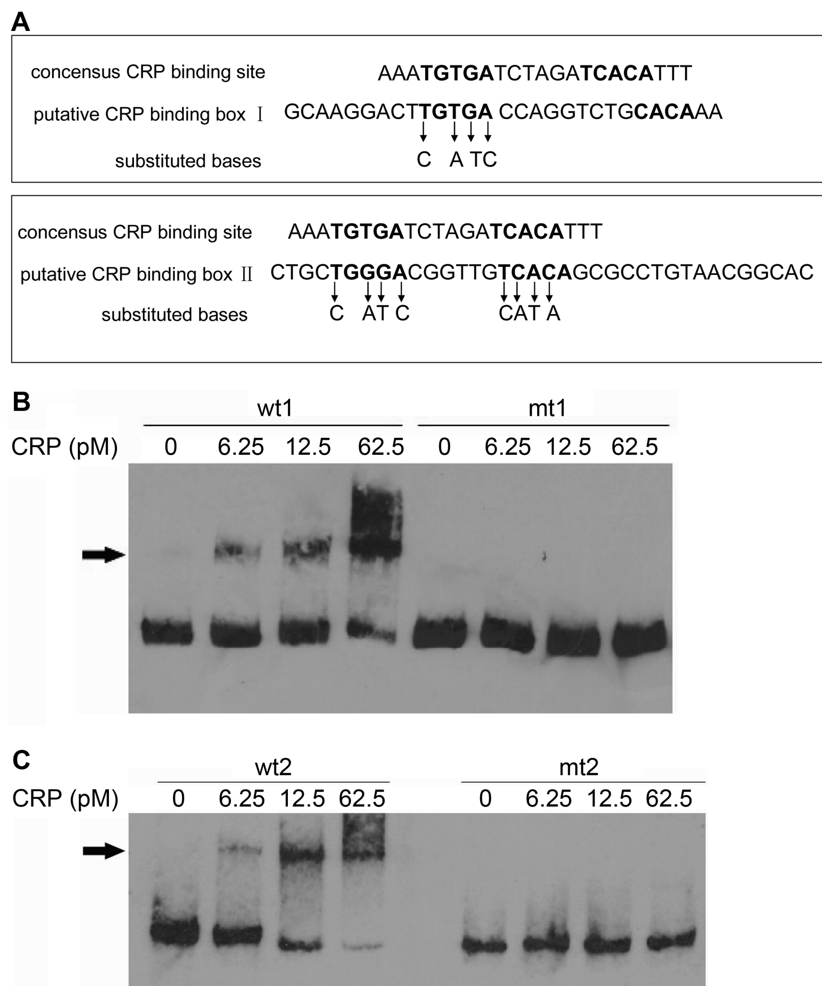

Fig. 4. cAMP−CRP specifically bound to two sites of the *rpoS* promoter *in vitro*.

A. Demonstration of consensus cAMP−CRP binding sequences and putative binding sites I and II in the *rpoS* promoter. The most consensus bases for binding are indicated by bold letter. The arrow indicates bases substituted for electrophoretic mobility shift assay (EMSA) and β-galactosidase activity assay. **B** and **C**. EMSA was performed as described in Materials and Methods. Labeled DNA fragments (20 fmole) of native sequences (wt1, wt2) and mutated sequences (mt1, mt2) were incubated with 0 to 62.5 pM CRP as indicated. cAMP was added in all reaction mixtures at a final concentration of 100 μM. The arrow denotes the cAMP−CRP−DNA complex.

shown that *katE* is regulated by *rpoS* [13, 23, 33]. Our study showed that there was no transcriptional change as to *katG* after deletion of *ppk* (data not shown), whereas transcription of *katE* was significantly increased (Fig. 2A). Correlating with the above observation, when exposed to H_2O_2, the *ppk* mutant showed higher resistance compared with WT (Fig. 2B). All these results suggest that *ppk* affects oxidative stress response by modulating *rpoS* transcription.

Dynamic accumulation of polyP is observed in *E. coli* when subjected to nutritional or osmotic stress or to nitrogen exhaustion, and the process is under regulation of ppGpp [3]. polyP is suggested to be an energy reservoir, and loss of *ppk* markedly increases the intracellular ATP level [26]. Considering these, we were curious about the possible role of polyP/*ppk* in the availability of intracellular cAMP−CRP complex, an important signal-receptor complex that is involved in the regulation of metabolism, flagellum synthesis, toxin production, and other cellular processes [4]. The cAMP−CRP complex is thought to be involved in the control of *rpoS* transcription, and its regulatory role has been investigated under various genetic backgrounds and growth conditions. Among them, the effect of *cya* or *crp* mutant is difficult to be interpreted owing to the growth deficiency of those mutants, considering the influence of growth rate on *rpoS* transcription [21, 24]. To explain the contradictory results in the literature, different roles of cAMP−CRP in a specific growth phase is proposed in *rpoS* transcription [10] but no experimental evidence can strongly support it hitherto. In this study, we observed the regulatory role of cAMP−CRP firstly through a direct binding assay *in vitro*. It was the first demonstration in enteric bacteria, that cAMP−CRP independently and

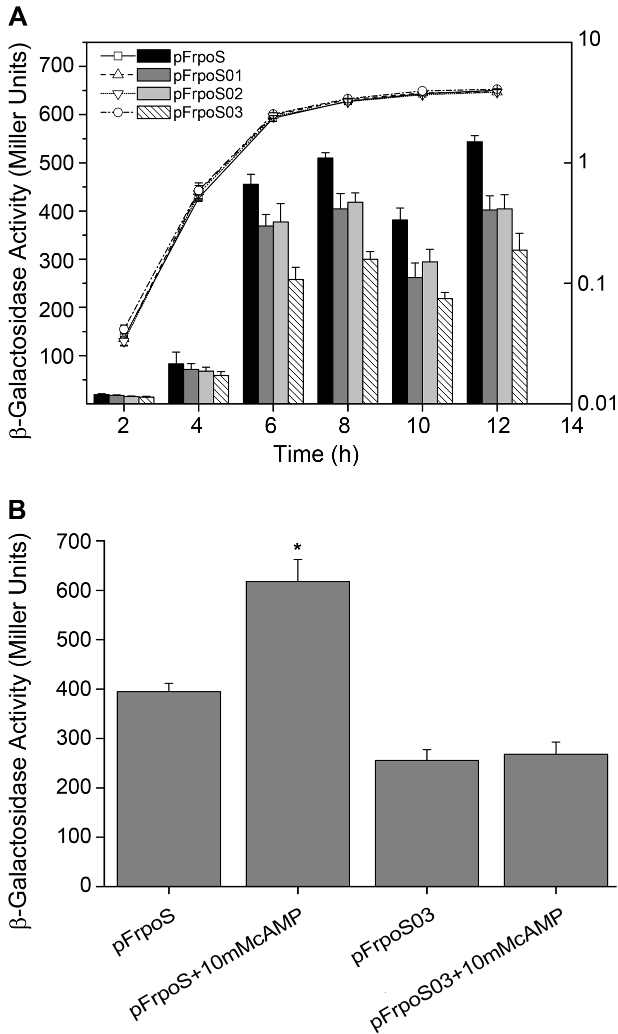

Fig. 5. cAMP−CRP stimulated transcription of *rpoS*.

A. The activity of the *rpoS* promoter with different mutations of CRP-binding sites. β-Galactosidase activity was monitored in WT carrying plasmids pFrpoS (fusing native *rpoS* promoter), pFrpoS01 (pFrpoS, except for mutation in CRP-binding site I), pFrpoS02 (pFrpoS, except for mutation in CRP-binding site II), and pFrpoS03 (pFrpoS, except for mutation in both sites). **B**. Exogenous cAMP-stimulated expression of *rpoS*. Overnight cultures of WT carrying pFrpoS or pFrpoS03 were diluted into fresh buffered LB with or without 10 mM cAMP to OD_{600} of 0.02. After growing for 12 h, β-galactosidase activity was measured. The asterisk indicates *P* values less than 0.01.

specifically bound to two sites of the *rpoS* promoter (Fig. 4). Then, base-substitutions were conducted in the two binding sites, within which the most consensus bases for cAMP−CRP binding were chosen in case of overlapping with other regulators' binding sites, and no consensus binding bases of other regulators known until now were affected. The mutations in one or both sites resulted in decreased transcription of *rpoS*, which indicated that both sites were responsible for stimulation of *rpoS* transcription in the late-exponential phase and stationary phase (Fig. 5A).

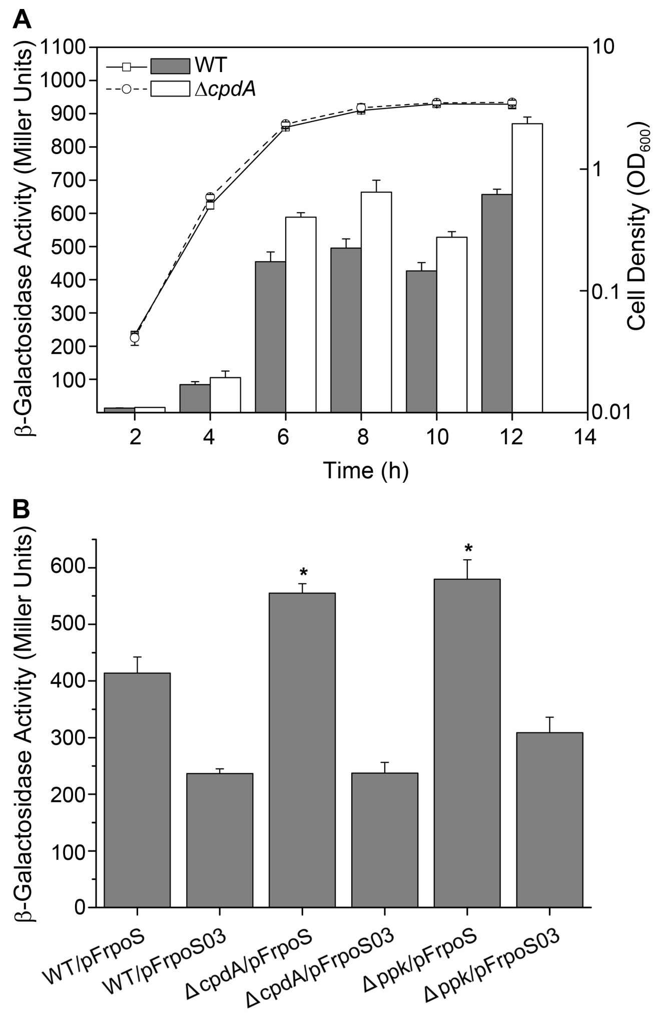

Fig. 6. A. The effect of *cpdA* deletion on thetranscription of *rpoS*.

Cells of WT and *cpdA* mutant (Δ*cpdA)* containing pFrpoS were cultured and subcultured as described in Materials and Methods. Aliquots were collected to measure OD_{600} and β-galactosidase activity. **B**. The activity of *rpoS* promoters with (pFrpoS03) and without (pFrpoS) mutation in both CRP-binding sites in WT, Δ*cpdA,* and Δ*ppk*. Overnight cultures were diluted and subcultured for 12 h to determine β-galactosidase activity.

Interestingly, in the stationary phase, we still observed a slight increase of *rpoS* transcription in the *ppk* mutant compared with WT, although both of the cAMP−CRP-binding sites were mutated (Fig. 6B). It suggests that, except for cAMP−CRP, additional factors may also be involved in the regulation of *rpoS* transcription and need to be identified in further research.

Acknowledgments

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References

1. Ahn, K. and A. Kornberg. 1990. Polyphosphate kinase from *Escherichia coli*. Purification and demonstration of a phosphoenzyme intermediate. *J. Biol. Chem*. **265:** 11734–11739.
2. Akiyama, M., E. Crooke, and A. Kornberg. 1993. An exopolyphosphatase of *Escherichia coli*. The enzyme and its *ppx* gene in a polyphosphate operon. *J. Biol. Chem*. **268:** 633–639.
3. Ault-Riche, D., C. D. Fraley, C. M. Tzeng, and A. Kornberg. 1998. Novel assay reveals multiple pathways regulating stress-induced accumulations of inorganic polyphosphate in *Escherichia coli*. *J. Bacteriol*. **180:** 1841–1847.
4. Botsford, J. L. and J. G. Harman. 1992. Cyclic AMP in prokaryotes. *Microbiol. Rev*. **56:** 100–122.
5. Campoy, S., M. Jara, N. Busquets, A. M. de Rozas, I. Badiola, and J. Barbe. 2002. Intracellular cyclic AMP concentration is decreased in *Salmonella* Typhimurium *fur* mutants. *Microbiology* **148:** 1039–1048.
6. Coynault, C., V. Robbe-Saule, and F. Norel. 1996. Virulence and vaccine potential of *Salmonella* Typhimurium mutants deficient in the expression of the RpoS (sigma S) regulon. *Mol. Microbiol*. **22:** 149–160.
7. Datsenko, K. A. and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U.S.A*. **97:** 6640–6645.
8. Fang, F. C., S. J. Libby, N. A. Buchmeier, P. C. Loewen, J. Switala, J. Harwood, and D. G. Guiney. 1992. The alternative sigma factor *katF* (*rpoS*) regulates *Salmonella* virulence. *Proc. Natl. Acad. Sci. U.S.A*. **89:** 11978–11982.
9. Giebel, L. B. and R. A. Spritz. 1990. Site-directed mutagenesis using a double-stranded DNA fragment as a PCR primer. *Nucleic Acids Res*. **18:** 4947.
10. Hengge-Aronis, R. 2002. Signal transduction and regulatory mechanisms involved in control of the sigma(S) (RpoS) subunit of RNA polymerase. *Microbiol. Mol. Biol. Rev*. **66:** 373–395, Table of Contents.
11. Hirsch, M. and T. Elliott. 2005. Fis regulates transcriptional induction of RpoS in *Salmonella enterica*. *J. Bacteriol*. **187:** 1568–1580.
12. Hirsch, M. and T. Elliott. 2002. Role of ppGpp in *rpoS* stationary-phase regulation in *Escherichia coli*. *J. Bacteriol*. **184:** 5077–5087.
13. Ibanez-Ruiz, M., V. Robbe-Saule, D. Hermant, S. Labrude, and F. Norel. 2000. Identification of RpoS (sigma(S))-regulated genes in *Salmonella enterica* serovar Typhimurium. *J. Bacteriol*. **182:** 5749–5756.
14. Imamura, R., K. Yamanaka, T. Ogura, S. Hiraga, N. Fujita, A. Ishihama, and H. Niki. 1996. Identification of the *cpdA* gene encoding cyclic 3',5'-adenosine monophosphate phosphodiesterase in *Escherichia coli*. *J. Biol. Chem*. **271:** 25423–25429.
15. Ivanova, A., C. Miller, G. Glinsky, and A. Eisenstark. 1994. Role of *rpoS* (*katF*) in *oxyR*-independent regulation of hydroperoxidase I in *Escherichia coli*. *Mol. Microbiol*. **12:** 571–578.
16. Jain, R. and S. Shuman. 2008. Polyphosphatase activity of CthTTM, a bacterial triphosphate tunnel metalloenzyme. *J. Biol. Chem*. **283:** 31047–31057.
17. Kim, H. S., S. M. Kim, H. J. Lee, S. J. Park, and K. H. Lee. 2009. Expression of the *cpdA* gene, encoding a 3',5'-cyclic AMP (cAMP) phosphodiesterase, is positively regulated by the cAMP–cAMP receptor protein complex. *J. Bacteriol*. **191:** 922–930.
18. Koop, A. H., M. E. Hartley, and S. Bourgeois. 1987. A low-copy-number vector utilizing beta-galactosidase for the analysis of gene control elements. *Gene* **52:** 245–256.
19. Kuroda, A., K. Nomura, R. Ohtomo, J. Kato, T. Ikeda, N. Takiguchi, *et al*. 2001. Role of inorganic polyphosphate in promoting ribosomal protein degradation by the Lon protease in *E. coli*. *Science* **293:** 705–708.
20. Lange, R., D. Fischer, and R. Hengge-Aronis. 1995. Identification of transcriptional start sites and the role of ppGpp in the expression of *rpoS*, the structural gene for the sigma S subunit of RNA polymerase in *Escherichia coli*. *J. Bacteriol*. **177:** 4676–4680.
21. Lange, R. and R. Hengge-Aronis. 1994. The cellular concentration of the sigma S subunit of RNA polymerase in *Escherichia coli* is controlled at the levels of transcription, translation, and protein stability. *Genes Dev*. **8:** 1600–1612.
22. Loewen, P. C., B. Hu, J. Strutinsky, and R. Sparling. 1998. Regulation in the *rpoS* regulon of *Escherichia coli*. *Can. J. Microbiol*. **44:** 707–717.
23. Loewen, P. C., J. Switala, and B. L. Triggs-Raine. 1985. Catalases HPI and HPII in *Escherichia coli* are induced independently. *Arch. Biochem. Biophys*. **243:** 144–149.
24. McCann, M. P., C. D. Fraley, and A. Matin. 1993. The putative sigma factor KatF is regulated posttranscriptionally during carbon starvation. *J. Bacteriol*. **175:** 2143–2149.
25. McInerney, P., T. Mizutani, and T. Shiba. 2006. Inorganic polyphosphate interacts with ribosomes and promotes translation fidelity *in vitro* and *in vivo*. *Mol. Microbiol*. **60:** 438–447.
26. McMeechan, A., M. A. Lovell, T. A. Cogan, K. L. Marston, T. J. Humphrey, and P. A. Barrow. 2007. Inactivation of *ppk* differentially affects virulence and disrupts ATP homeostasis in *Salmonella enterica* serovars Typhimurium and Gallinarum. *Res. Microbiol*. **158:** 79–85.
27. Mika, F. and R. Hengge. 2005. A two-component phosphotransfer network involving ArcB, ArcA, and RssB coordinates synthesis and proteolysis of sigmaS (RpoS) in *E. coli*. *Genes Dev*. **19:** 2770–2781.
28. Miller, J. H. 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. Nickerson, C. A. and R. Curtiss, 3rd. 1997. Role of sigma factor RpoS in initial stages of *Salmonella* Typhimurium infection. *Infect. Immun*. **65:** 1814–1823.
30. Nomura, K., J. Kato, N. Takiguchi, H. Ohtake, and A. Kuroda. 2004. Effects of inorganic polyphosphate on the proteolytic and DNA-binding activities of Lon in *Escherichia coli*. *J. Biol. Chem*. **279:** 34406–34410.
31. Paesold, G. and M. Krause. 1999. Analysis of *rpoS* mRNA in *Salmonella* Dublin: Identification of multiple transcripts with growth-phase-dependent variation in transcript stability. *J. Bacteriol*. **181:** 1264–1268.
32. Rashid, M. H., K. Rumbaugh, L. Passador, D. G. Davies, A. N. Hamood, B. H. Iglewski, and A. Kornberg. 2000. Polyphosphate kinase is essential for biofilm development, quorum sensing, and virulence of *Pseudomonas aeruginosa*. *Proc. Natl. Acad. Sci. U.S.A*. **97:** 9636–9641.
33. Schellhorn, H. E. and H. M. Hassan. 1988. Transcriptional regulation of *katE* in *Escherichia coli* K-12. *J. Bacteriol*. **170:** 4286–4292.

34. Shiba, T., K. Tsutsumi, H. Yano, Y. Ihara, A. Kameda, K. Tanaka, *et al*. 1997. Inorganic polyphosphate and the induction of *rpoS* expression. *Proc. Natl. Acad. Sci. U.S.A*. **94:** 11210–11215.
35. Stumpf, J. D. and P. L. Foster. 2005. Polyphosphate kinase regulates error-prone replication by DNA polymerase IV in *Escherichia coli*. *Mol. Microbiol*. **57:** 751–761.
36. Sureka, K., S. Dey, P. Datta, A. K. Singh, A. Dasgupta, S. Rodrigue, *et al*. 2007. Polyphosphate kinase is involved in stress-induced mprAB-sigE-rel signalling in mycobacteria. *Mol. Microbiol*. **65:** 261–276.
37. Takayanagi, Y., K. Tanaka, and H. Takahashi. 1994. Structure of the 5' upstream region and the regulation of the *rpoS* gene of *Escherichia coli*. *Mol. Gen. Genet*. **243:** 525–531.
38. Visick, J. E. and S. Clarke. 1997. RpoS- and OxyR-independent induction of HPI catalase at stationary phase in *Escherichia coli* and identification of *rpoS* mutations in common laboratory strains. *J. Bacteriol*. **179:** 4158–4163.
39. Weber, H., T. Polen, J. Heuveling, V. F. Wendisch, and R. Hengge. 2005. Genome-wide analysis of the general stress response network in *Escherichia coli*: Sigmas-dependent genes, promoters, and sigma factor selectivity. *J. Bacteriol*. **187:** 1591–1603.
40. Weichart, D., R. Lange, N. Henneberg, and R. Hengge-Aronis. 1993. Identification and characterization of stationary phase-inducible genes in *Escherichia coli*. *Mol. Microbiol*. **10:** 407–420.