AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology®

RESEARCH ARTICLE

Basal-Level Effects of (p)ppGpp in the Absence of Branched-Chain Amino Acids in *Actinobacillus pleuropneumoniae*

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ABSTRACT The (p)ppGpp-mediated stringent response (SR) is a highly conserved regulatory mechanism in bacterial pathogens, enabling adaptation to adverse environments, and is linked to pathogenesis. *Actinobacillus pleuropneumoniae* can cause damage to the lungs of pigs, its only known natural host. Pig lungs are known to have a low concentration of free branched-chain amino acids (BCAAs) compared to the level in plasma. We had investigated the role for (p)ppGpp in viability and biofilm formation of *A. pleuropneumoniae*. Now, we sought to determine whether (p)ppGpp was a trigger signal for the SR in *A. pleuropneumoniae* in the absence of BCAAs. Combining transcriptome and phenotypic analyses of the wild type (WT) and an *relA spoT* double mutant [which does not produce (p)ppGpp], we found that (p)ppGpp could repress *de novo* purine biosynthesis and activate antioxidant pathways. There was a positive correlation between GTP and endogenous hydrogen peroxide content. Furthermore, the growth, viability, morphology, and virulence were altered by the inability to produce (p)ppGpp. Genes involved in the biosynthesis of BCAAs were constitutively upregulated, regardless of the existence of BCAAs, without accumulation of (p)ppGpp beyond a basal level. Collectively, our study shows that the absence of BCAAs was not a sufficient signal to trigger the SR in *A. pleuropneumoniae*. (p)ppGpp-mediated regulation in *A. pleuropneumoniae* is different from that described for the model organism *Escherichia coli*. Further work will establish whether the (p)ppGpp-dependent SR mechanism in *A. pleuropneumoniae* is conserved among other veterinary pathogens, especially those in the *Pasteurellaceae* family.

IMPORTANCE (p)ppGpp is a key player in reprogramming transcriptomes to respond to nutritional challenges. Here, we present transcriptional and phenotypic differences of *A. pleuropneumoniae* grown in different chemically defined media in the absence of (p)ppGpp. We show that the deprivation of branched-chain amino acids (BCAAs) does not elicit a change in the basal-level (p)ppGpp, but this level is sufficient to regulate the expression of BCAA biosynthesis. The mechanism found in *A. pleuropneumoniae* is different from that of the model organism *Escherichia coli* but similar to that found in some Gram-positive bacteria. This study not only broadens the research scope of (p)ppGpp but also further validates the complexity and multiplicity of (p)ppGpp regulation in microorganisms that occupy different biological niches.

KEYWORDS *Actinobacillus pleuropneumoniae*, stringent response, BCAAs, (p)ppGpp, GTP

Citation Li G, Zhao Q, Luan T, Hu Y, Zhang Y, Li T, Wang C, Xie F, Zhang W, Langford PR, Liu S. 2020. Basal-level effects of (p)ppGpp in the absence of branched-chain amino acids in *Actinobacillus pleuropneumoniae*. J Bacteriol 202:e00640-19. https://doi.org/10.1128/JB.00640-19.

Editor Laurie E. Comstock, Brigham and Women's Hospital/Harvard Medical School

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Received 8 October 2019
Accepted 24 January 2020
Accepted manuscript posted online 3 February 2020
Published 26 March 2020

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The alarmone (p)ppGpp was first identified 50 years ago as accumulating in response to amino acid starvation in *Escherichia coli* (1). Mounting evidence has indicated that (p)ppGpp is a key factor in bacterial physiology, including the rapid response to diverse stresses, which profoundly affects cellular processes, including transcription, replication, and translation, and is important for virulence (2, 3), differentiation, and persistence (4–7). The (p)ppGpp-induced starvation response is called the stringent response (SR), and this signaling pathway is utilized by the vast majority of bacterial species to mediate the stress response to amino acid starvation (8–10). The RelA/SpoT homologue (RSH) proteins, which synthesize or degrade (p)ppGpp, have been found throughout the tree of life (11, 12). There are three main groups of enzymes in the RSH superfamily: long RSH enzymes, small alarmone synthetases (SAS), and small alarmone hydrolases (SAH).

In most beta- and gammaproteobacteria, (p)ppGpp is synthesized by the monofunctional synthetase RelA and degraded by the bifunctional protein SpoT (13). While there are good data on the role of (p)ppGpp in growth regulation with the model microorganisms *Escherichia coli* and *Bacillus subtilis* (14–21), there is comparatively less information for bacterial pathogens of veterinary origin.

Actinobacillus pleuropneumoniae is a gammaproteobacterium which is the causative agent of porcine pleuropneumonia, a highly contagious respiratory disease that causes significant economic losses throughout the worldwide porcine industry (22). Typically, it is isolated from the swine respiratory tract (23, 24), with acute and chronic disease caused by *A. pleuropneumoniae* being associated with lung tissue damage. Field isolates are classified into two biotypes depending on their requirement for NAD and further subdivided into 18 serovars based on capsular antigens (25–27). Some major virulence factors required for the development of clinical disease have been identified, e.g., the RTX toxins, iron acquisition proteins, and components of pathways required for survival in anaerobic environments (reviewed in reference 23). In addition, an *A. pleuropneumoniae* knockout mutant of *ilvI*, which encodes the enzyme acetohydroxy-acid synthase (AHAS) required for branched-chain amino acid (BCAA) biosynthesis, was attenuated for virulence in pigs (28, 29), reflecting that the BCAA content in porcine pulmonary epithelial lining fluid is only 10% to 17% of the concentration in plasma (28, 29). Whether the low availability of BCAAs in the porcine lung is a signal that triggers the SR in *A. pleuropneumoniae* is unknown, and there are relatively little data from other pathogens (30–32). The aim of this study was to determine whether there was a link between BCAA availability and the SR in *A. pleuropneumoniae* and the effect on phenotypic characteristics.

In our previous work, we showed that deletion of *A. pleuropneumoniae relA* alone had phenotypic effects, e.g., on the ability to form biofilms (33). In this study, we have additionally constructed a *relA spoT* double mutant [here, (p)ppGpp^0] that does not produce (p)ppGpp and compared the global transcriptional profile with that of the wild type (WT) in chemically defined medium (CDM) and determined phenotypic characteristics. The transcriptome results strongly indicate that the basal level of (p)ppGpp blocks *de novo* synthesis of purine nucleotides; there were significant changes in gene expression levels in antioxidant metabolic pathways, and the balance of GTP homeostasis and H_2O_2 content was altered. Thus, in *A. pleuropneumoniae*, the basal level of (p)ppGpp appears to constitutively regulate the transcription of genes involved in biosynthesis of BCAAs and purine. Interestingly, the direction of regulation of these two pathways is opposite from that described in the model organism *E. coli* (34). Thus, researchers are advised to be cautious in extrapolating data obtained with model microorganisms to their specific pathogen of interest.

RESULTS

The growth characteristics of the WT and (p)ppGpp^0 are the same in rich medium, but (p)ppGpp^0 growth is impaired upon sudden starvation. First, we tested the ability of WT and mutant strains to synthesize (p)ppGpp. Quantification of extracted nucleotides separated by thin-layer chromatography (TLC) (Fig. 1A) indicated that no detect-

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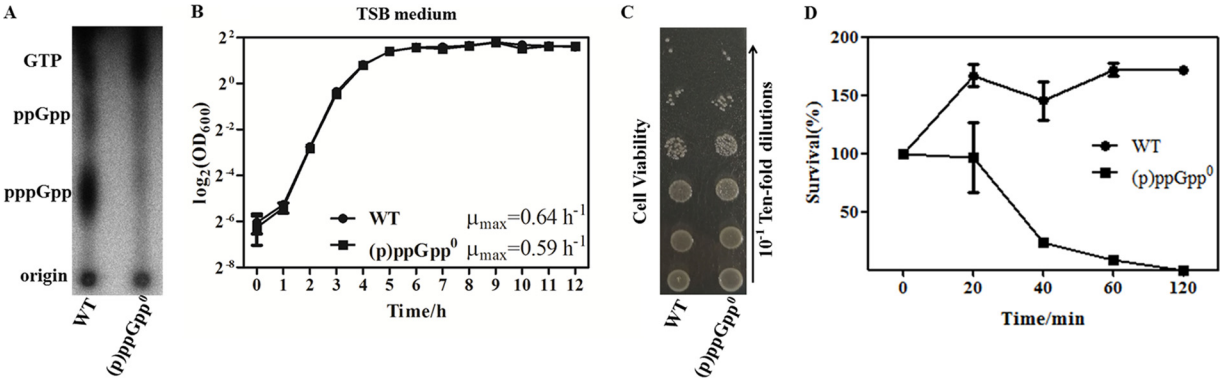


FIG 1 Synthesis of (p)ppGpp and growth profiles of the WT and (p)ppGpp^0 grown in rich medium. (A) Accumulation of (p)ppGpp in the WT and (p)ppGpp^0 mutant. Cells were labeled with [^{32}P]H_3PO_4 in MOPS under starvation conditions; the formic acid extracts of the cells were subjected to TLC analysis as described in Materials and Methods. (B) Growth curves of the WT and (p)ppGpp^0 mutant. Growth was monitored by OD_{600} at various time points. (C) Cell viability of the WT and (p)ppGpp^0 mutant. Overnight cultures were serially diluted with fresh medium and spotted onto the TSA plate. (D) (p)ppGpp^0 failed to survive sudden starvation. The WT and (p)ppGpp^0 mutant were treated with 0.5 mg/ml RHX for the indicated times and plated on TSA. Percent survival was calculated by counting the numbers of colonies and normalized to the number at time zero. All data are shown as arithmetic means and standard deviations from three replicates.

able (p)ppGpp was produced in the (p)ppGpp^0 mutant. No significant difference in the growth rates and in levels of viability was observed between the WT and (p)ppGpp^0 (Fig. 1B and C). However, when the (p)ppGpp^0 mutant was subjected to sudden amino acid starvation, its viability decreased sharply, unlike results with the WT. Within 120 min of amino acid starvation, only 8% of (p)ppGpp^0 cells had survived, whereas 160% of WT cells survived (Fig. 1D). The result indicates that (p)ppGpp is required for the WT to survive amino acid starvation, i.e., nutrient stress.

(p)ppGpp is required for the maintenance of proper growth and viability in the absence of BCAAs. That the alarmone (p)ppGpp has a major role in regulating growth of bacteria has been reported elsewhere (4, 5, 16). In order to determine the precise amino acid requirement of *A. pleuropneumoniae*, we examined its growth in 20 media, i.e., normal CDM and CDM lacking each amino acid. The results indicate that (p)ppGpp^0 has a strong requirement for isoleucine and valine and a weaker requirement for leucine for growth under the conditions tested (see Fig. S1 in the supplemental material). Growth curve and viability data from both the WT and the (p)ppGpp^0 mutant in CDM with deletion of a BCAA (CDM-BCAA) suggest that the WT grows better in some amino acid-deleted media than in CDM (Fig. 2A and C). However, loss of (p)ppGpp resulted in decreased growth and viability regardless of the existence of BCAAs (Fig. 2B and D). To determine whether BCAAs acted as a starvation signal sufficient enough to be sensed by *A. pleuropneumoniae*, we determined the formation of (p)ppGpp in the WT grown in various media. Our results show that deleting BCAAs from CDM did not increase the production (p)ppGpp; the basal level in *A. pleuropneumoniae* WT remained unchanged (Fig. 2E).

(p)ppGpp production is associated with bacterial morphological changes. We determined whether (p)ppGpp was associated with changes in *A. pleuropneumoniae* morphology as determined by transmission electron microscopy. In nutrient-rich tryptic soy broth (TSB) medium, the morphologies of the WT and (p)ppGpp^0 were similar (Fig. 3). However, in CDM and BCAA-deleted CDM, there were differences in bacterial morphologies. The WT structure was similar in rich medium to that observed in BCAA-deleted CDM. In contrast, in BCAA-depleted CDM, the (p)ppGpp^0 mutant had fewer septa than the WT. Additionally, the smoothness of the surface appeared decreased, the cells were longer, and some bacteria were severely deformed. Such changes were most apparent when (p)ppGpp^0 was cultured in CDM lacking valine (CDM-valine). These results suggest an association between phenotype and the (p)ppGpp-mediated SR.

Availability of (p)ppGpp affects *A. pleuropneumoniae* virulence. In order to assess the contribution of (p)ppGpp to virulence, we determined the 50% lethal dose

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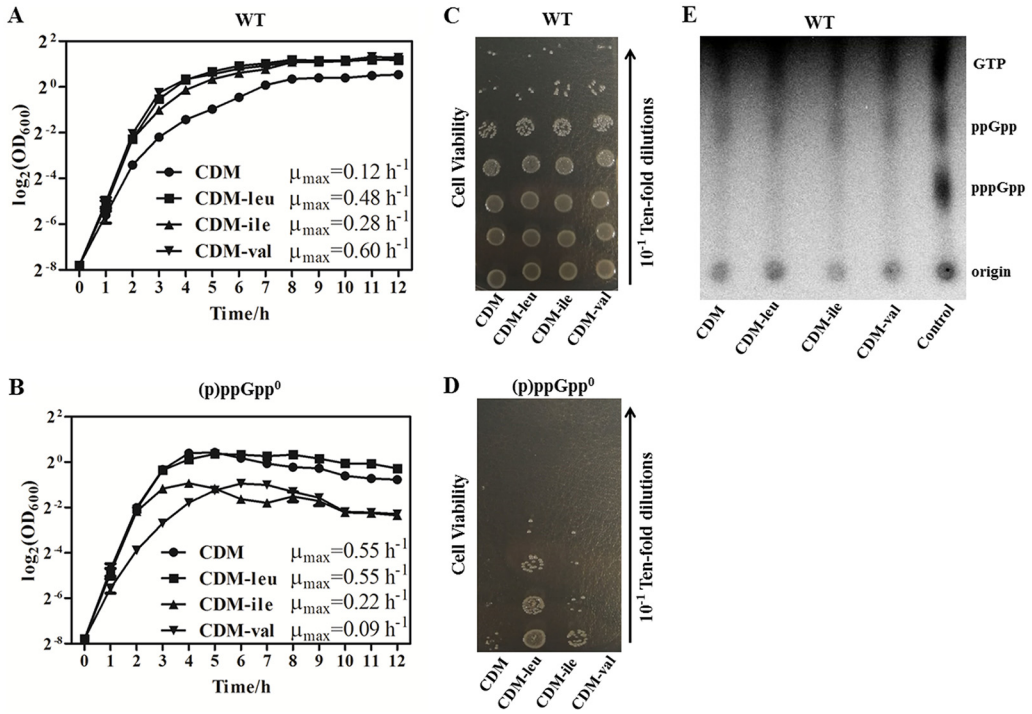


FIG 2 Growth and accumulation of the (p)ppGpp in WT and (p)ppGpp^0 mutant under different growth conditions. (A and B) Growth curves of the WT and (p)ppGpp^0 mutant in CDM and CDM-BCAA. The value of the OD_{600} was monitored at various time points. (C and D) The viability of the WT and (p)ppGpp^0 mutant in CDM and CDM-BCAA. Overnight cultures were serially diluted by fresh medium and spotted onto the TSA plate. (E) Determination of the production of (p)ppGpp by the WT in CDM and CDM-BCAA. Values shown in panels A and B are arithmetic means and standard deviations of results from at least three independent experiments.

(LD_{50}) of the WT and (p)ppGpp^0 strain for larvae of *Galleria mellonella* (35). The result suggested that the absence of (p)ppGpp weakens the virulence of the (p)ppGpp^0 mutant by 2-fold (Fig. S2).

Overview of transcriptome differences between the WT and (p)ppGpp^0 grown in CDM or CDM-BCAA. To gain a more thorough understanding of the genetic basis, transcriptome analysis was done to delineate the mechanisms underlying (p)ppGpp-mediated function under BCAA-deleted conditions. First, we compared the transcriptome of the WT which was cultured with or without BCAAs in CDM (Fig. 4A, upper table). Unexpectedly, there was little difference in transcriptomes. Similar results were obtained with the (p)ppGpp^0 mutant (Fig. 4A, upper table). This result supports the

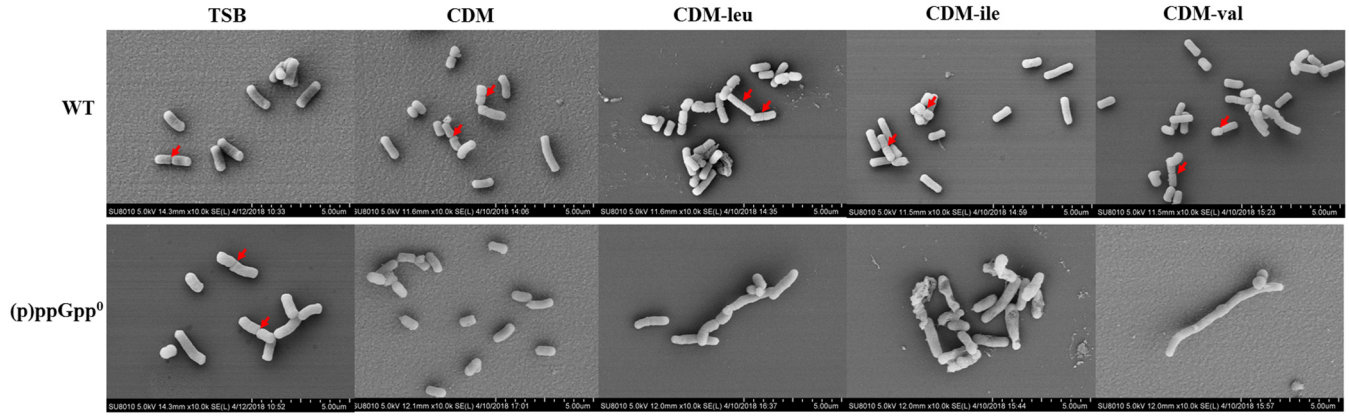
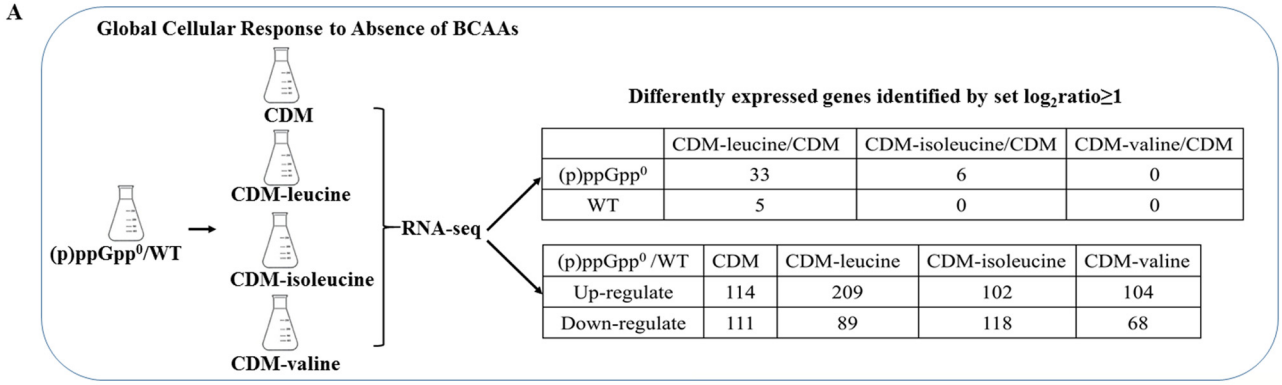


FIG 3 Morphology of the *A. pleuropneumoniae* WT and (p)ppGpp^0 mutant grown in different media as determined by scanning electron microscopy.

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	CDM-leucine/CDM	CDM-isoleucine/CDM	CDM-valine/CDM
(p)ppGpp^0	33	6	0
WT	5	0	0

(p)ppGpp^0 /WT	CDM	CDM-leucine	CDM-isoleucine	CDM-valine
Up-regulate	114	209	102	104
Down-regulate	111	89	118	68

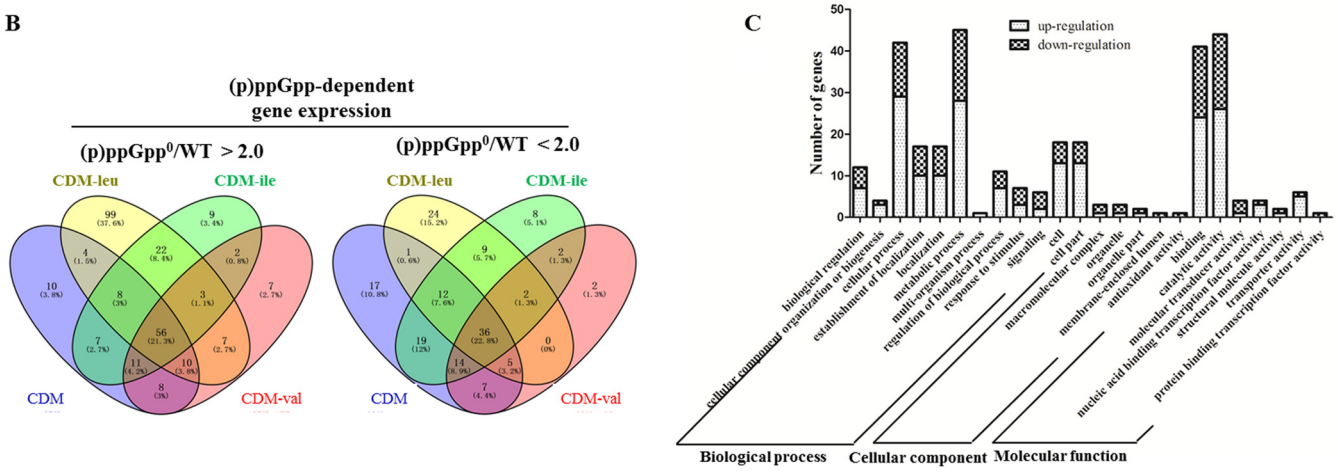


FIG 4 Transcriptome analysis of (p)ppGpp-dependent genes during growth in CDM or CDM-BCAA. (A) Schematic of differentially expressed genes affected by (p)ppGpp in different media. (B) Venn diagrams showing the number of genes that are upregulated and downregulated in a (p)ppGpp^0-dependent manner under CDM and CDM-BCAA growth conditions. (C) Functional categories of genes differentially expressed in (p)ppGpp^0 compared with the levels in the WT in the absence of BCAAs. The number of genes whose expression is differentially expressed in (p)ppGpp^0 compared with the level in the WT is presented according to the functions assigned by the browser of CLRNA-Seq data analysis software.

observation that the WT did not induce accumulation of (p)ppGpp in the absence of single BCAAs. Although the absence of BCAAs had little effect on the transcriptome of the WT and (p)ppGpp^0 mutant, the response to (p)ppGpp was quite different when the WT and the (p)ppGpp^0 mutant were grown in the same medium. When the (p)ppGpp^0 mutant was compared to the WT in normal CDM (see Data Set S1 in the supplemental material), there were 114 upregulated and 111 downregulated genes (Fig. 4A, bottom table). There were 209 upregulated and 89 downregulated genes in the (p)ppGpp^0 mutant compared to levels in the WT when the two strains were cultured in CDM-leucine medium (Fig. 4A, bottom table). There were 118 upregulated and 102 downregulated genes in the (p)ppGpp^0 mutant compared to levels in the WT when both were cultured in CDM-isoleucine medium (Fig. 4A, bottom table). There were 104 upregulated and 68 downregulated genes in the (p)ppGpp^0 mutant compared to levels in the WT when both were cultured in CDM-valine medium (Fig. 4A, bottom table).

Among these differentially expressed genes, there were 56 upregulated and 36 downregulated in common under CDM and CDM-BCAA conditions compared to levels in the WT (Fig. 4B). Differentially expressed genes were annotated according to the Clusters of Orthologous Groups (COG) database and are summarized in Fig. 4C. The complete list of these genes is shown in Data Set S1 in the supplemental material.

The gene expression profiles identified some common and some different processes controlled by (p)ppGpp. We found that abundant genes associated with the intracellular ribonucleoprotein complex, ribosome biogenesis, membrane proteins, and the purine biosynthesis pathway were all upregulated in the (p)ppGpp^0 mutant. In contrast, some genes involved in metal transport and antioxidant pathways were downregu-

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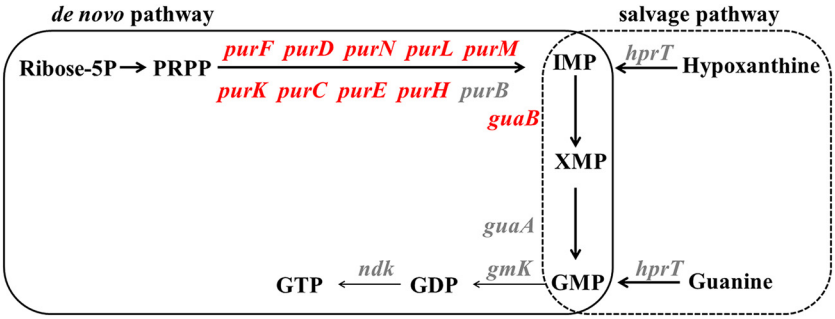


FIG 5 Schematic of the pathways affected by (p)ppGpp. Starvation-induced changes in the purine biosynthesis pathway. The indicated genes are involved in the purine *de novo* and salvage biosynthesis pathways. Red, genes with more than 2-fold upregulation; gray, genes with no detectable change in transcript levels.

lated. The expression levels of all of these genes were the opposite of those found in the WT. This suggests that the lack of (p)ppGpp leads to a transcriptionally relaxed state in the (p)ppGpp^0 mutant. Finally, the transcriptional misregulation results in the slow growth and decreased viability of the (p)ppGpp^0 mutant.

We also found that the genes encoding the acetohydroxy acid synthase (AHAS) isozyme (*ilvIHM*; genes B838_RS0101105, B838_RS0101110, and B838_RS0108605), catalyzing the first step in the biosynthesis of the BCAAs, were all inhibited in (p)ppGpp^0 regardless of the presence or absence of BCAA.

Additionally, the *zapB* (B838_RS0106965) and *ftsA* (B838_RS0102230) genes, which are required for cell division and proper Z-ring formation, were upregulated in (p)ppGpp^0 except in CDM-valine medium. This may explain the filamentous phenotype of (p)ppGpp^0 in the absence of valine in CDM.

A response regulator (B838_RS0102415) of a two-component system involved in the regulation of nitrate metabolism was also downregulated. Two genes (B838_RS11255 and B838_RS0100090) that are associated with lysine biosynthesis were inhibited more than 4-fold. Another three hypothetical proteins (B838_RS0105755, B838_RS0105760, and B838_RS0100880) were also significantly downregulated. All of these different genes may also be putative targets of (p)ppGpp.

The purine nucleotide *de novo* biosynthesis pathways are activated in (p)ppGpp^0 including in CDM. Enrichment analysis among the 56 commonly upregulated genes in the (p)ppGpp^0 mutant indicated 10 genes (*purF*, *purD*, *purN*, *purL*, *purM*, *purK*, *purC*, *purH*, *purE*, and *guaB*) involved in the *de novo* purine biosynthesis and salvage pathways to be significantly upregulated (Fig. 5). Their action results in synthesis of IMP from phosphoribosyl pyrophosphate (PRPP), which is a key intermediate in the synthesis of purine nucleoside triphosphates GTP and ATP. However, there were no detectable differences in gene expression levels of *guaA*, *gmk*, and *hprT*. GuaA and Gmk are involved in pathways downstream from IMP to GTP. Hypoxanthine phosphoribosyltransferase (HprT) is a key enzyme of the salvage pathway, which converts purines with similar chemical structures to GMP and IMP, respectively.

Our results demonstrated that there was gene repression upstream in the pathways necessary for IMP biosynthesis. Combining previous observations, (p)ppGpp may affect multiple layers of regulation at the transcriptional level directly or possibly via post-transcriptional control. Our data suggest that in the absence of the *relA* and *spoT* genes in the (p)ppGpp^0 mutant, the metabolic flux progresses toward the accumulation of GTP, and this results in the disruption of GTP homeostasis directly. Finally, we identified the promoter motif associated with regulation of genes involved in the *A. pleuropneumoniae* purine biosynthesis pathway (Fig. 6; Data Set S2). The results indicate that these promoter motifs contain more A and T nucleotides than the (p)ppGpp-repressed promoters reported in *E. coli* and are similar to (p)ppGpp-activated promoters (34). This suggests that the sequence preference recognized by the transcription factor of *A. pleuropneumoniae* is different from that in *E. coli*.

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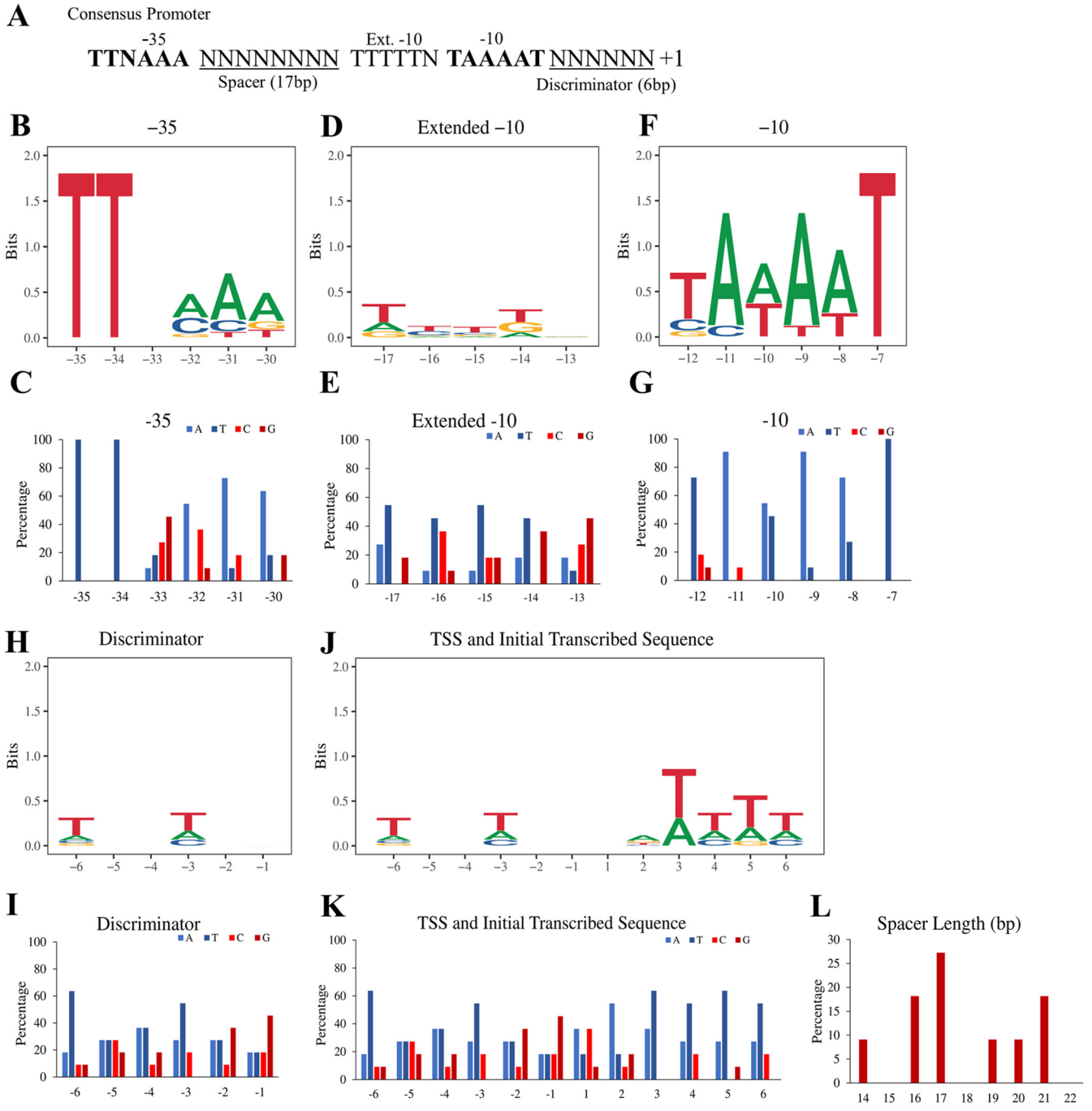


FIG 6 Sequence characteristics of promoters regulated by transcription factors. (A) Consensus promoter elements. Sequence conservation in the −35 element (B and C), extended −10 region (D and E), −10 element (F and G), discriminator element (H and I), transcription start site (TSS) region (J and K), and −10/−35 spacer length (L). Promoters of genes involved in purine biosynthesis pathway were aligned to create sequence logos (B, D, F, H, and J) and histograms of base distributions (C, E, G, J, I, K, and L) for each position.

The ROS metabolic pathways are repressed in (p)ppGpp⁰. Among the genes that were commonly downregulated in the (p)ppGpp⁰ mutant compared to the levels in the WT after growth in CDM and CDM-BCAA, most were classified as involved in amino acid, nucleotide, and carbohydrate metabolism and in environmental information processing (Fig. 4C). Four important genes that are related to antioxidants were substantially downregulated: superoxide dismutase (Fe-Mn family) (B838_RS0109035, $\log_2$ ratio = −1.34 ± 0.19), thioredoxin-disulfide reductase (B838_RS0108535, $\log_2$ ratio = −1.16 ±

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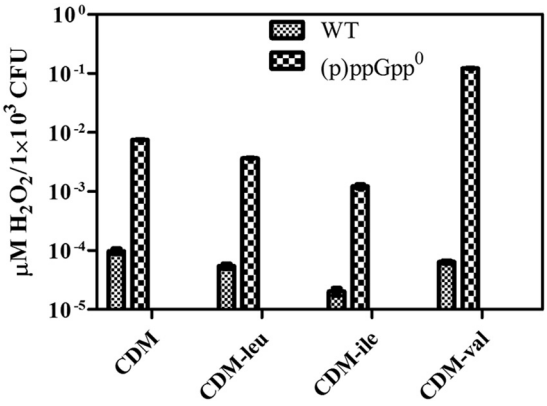


FIG 7 Enhanced H_2O_2 production by the (p)ppGpp^0 mutant. The cells of the WT and (p)ppGpp^0 mutant grown in CDM and CDM-BCAA were harvested and washed in PBS. The washed cell suspensions and medium were mixed with an equal volume of buffer to determine cytoplasmic H_2O_2 production, according to the instructions of an H_2O_2-peroxidase assay kit.

0.10), 2-Cys peroxiredoxin (B838_RS0110750, $\log_2$ ratio = −1.84 ± 0.22), and oxidoreductase (B838_RS0107845, $\log_2$ ratio = −1.19 ± 0.12). The downregulation of antioxidant genes would lead to an increase in reactive oxygen species (ROS) levels *in vivo*. ROS are formed as a natural by-product of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis. *A. pleuropneumoniae* is a facultative anaerobe, but superoxide radicals and hydrogen peroxide (H_2O_2) can be generated during aerobic amino acid metabolism (36). ROS are considered a primary source of intracellular oxidative stress and can cause significant damage to cells. Our transcriptome results suggest that transcriptional inhibition of superoxide dismutase in the (p)ppGpp^0 mutant will most likely result in an increase in exposure to superoxide radicals, which in turn will affect cell growth.

The levels of H_2O_2 support the altered transcriptome. The above observations led us to reason that downregulation of the expression of antioxidant genes would result in H_2O_2 accumulation. To investigate whether (p)ppGpp could affect the production of H_2O_2, we compared the intracellular concentrations of H_2O_2 in the WT and (p)ppGpp^0 mutant in CDM and CDM-BCAA. The results show that there is more than a 100-fold increase in cytoplasmic H_2O_2 production in (p)ppGpp^0 compared to the level in the WT (Fig. 7). H_2O_2 content is inversely related to CFU counts. We also found that the WT produces less intracellular H_2O_2 when BCAAs are absent from the normal CDM. The increased H_2O_2 production in the (p)ppGpp^0 mutant suggests that the ROS balance was significantly negatively affected and may be one of the main reasons for the decrease in viability in stationary phase. This result also suggested that loss of (p)ppGpp impairs protection against superoxide-mediated toxicity during stationary phase.

The relationship between GTP/H_2O_2 level and viability. To test the causal relationship between GTP levels and viability, we treated cells with the GMP synthase (GuaA) inhibitor decoyinine (Dec) to inhibit GTP biosynthesis and found that the growth and viability of both the WT (Fig. 8A and C) and (p)ppGpp^0 mutant (Fig. 8B and D) were inhibited. However, decoyinine seemed to delay a rapid decline in the number of viable cells in the (p)ppGpp^0 mutant in the stationary phase (Fig. 8D). In contrast, guanosine (Guo; 5 mM) addition to CDM, which increases GTP levels by the purine salvage pathway, significantly enhanced growth of the WT in stationary phase (Fig. 8C). The survival of (p)ppGpp^0 decreased compared to that of the WT strain (Fig. 8D). There appears to be a positive correlation between the GTP level and growth for the WT. However, the viability of the (p)ppGpp^0 mutant in stationary phase showed a negative correlation with GTP level. At the same time, high GTP levels reduced the accumulation of H_2O_2 in the WT (Fig. 8E). This relationship seems to be broken due to the lack of (p)ppGpp in the (p)ppGpp^0 mutant.

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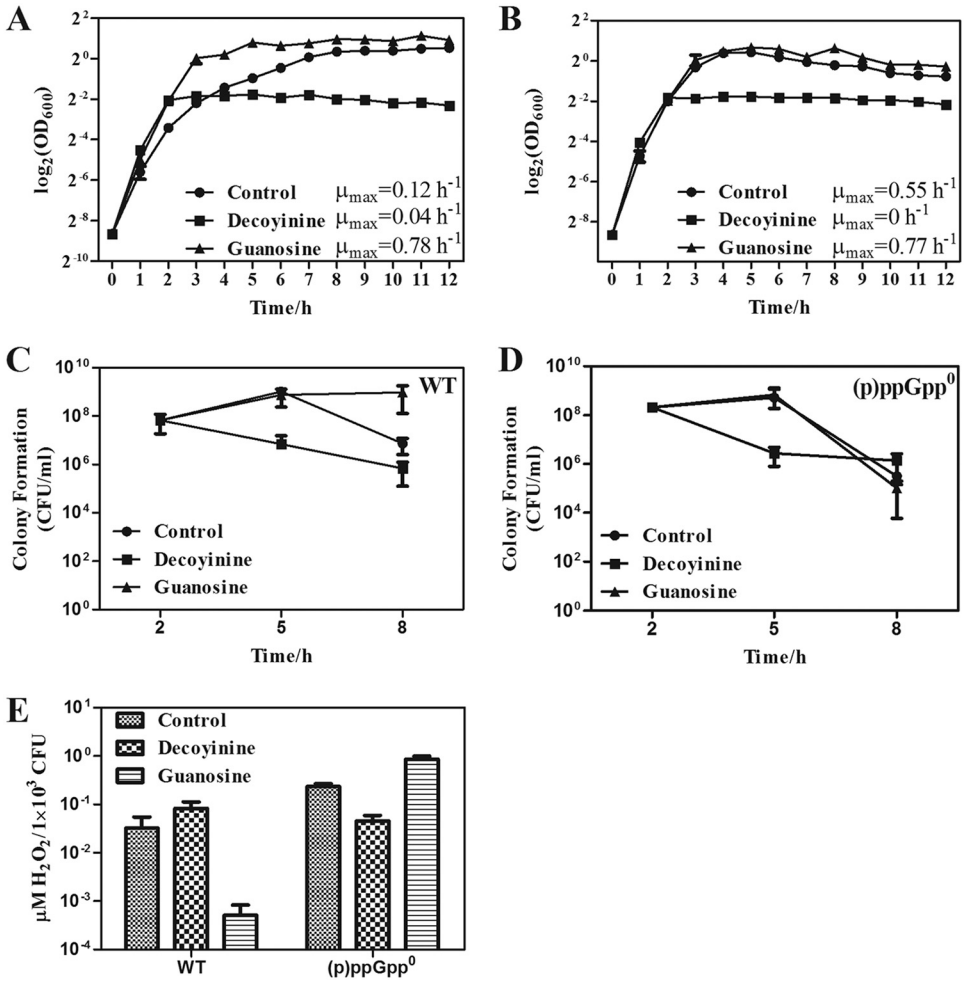


FIG 8 Growth, viability, and H_2O_2 accumulation affected by addition of decoyinine and guanosine. Cells were grown to early log phase (2 h) in CDM and supplemented with 5 mM guanosine and 50 μg/ml decoyinine. (A and B) The growth curves of the WT and (p)ppGpp^0 mutant were determined by monitoring the value of the OD_{600} at various time points. (C and D) The viability of the WT and (p)ppGpp^0 mutant was determined by counting the CFU at various time points. (E) The accumulation of H_2O_2 was determined by using a H_2O_2-peroxidase assay kit.

DISCUSSION

In our previous study, we evaluated the role of the *relA* gene in regulating biofilm formation and viability (33). In this study, in order to exclude the synthase activity of SpoT, which can produce (p)ppGpp in response to diverse stress signals (37), we constructed an *A. pleuropneumoniae relA spoT* (Δ*relA*Δ $spoT_{App}$) double mutant. We found that there was no significant difference in the growth rates and levels of viability of the WT and the (p)ppGpp^0 mutant in nutrient-rich medium. Subsequently, we cultured the WT and (p)ppGpp^0 strains in medium with or without BCAAs. Under such growth conditions, the growth rate, morphology, and viability of the (p)ppGpp^0 mutant were affected severely.

To explain the observed phenomena, we carried out transcriptional analyses and found that the presence or absence of BCAAs had little effect on the transcriptomes of the WT or the (p)ppGpp^0 mutant when they were cultured in different media, supporting the growth phenotypes observed in this study.

However, significant changes were seen when the transcriptomes of the WT and (p)ppGpp^0 mutant grown in the same medium were compared. Ten genes involved in the *de novo* and salvage pathways of purine nucleotide synthesis were repressed in the WT. In other bacteria, the *gmk* and *hprT* genes have been proved that its regulation occurs posttranscriptionally (38–41). (p)ppGpp could inhibit Gmk and HprT directly; this

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may explain why there are no changes in transcript levels for these two genes. Recently, some putative (p)ppGpp targets have been identified in *E. coli* (42, 43). These include PurF, which catalyzes the first step of GTP biosynthesis pathway, and the proteins encoded by the *purC* and *guaB* genes, both differentially regulated in this study. In general, except for the *guaA*, *gmk*, and *hprT* genes which were not differentially expressed, the transcription of genes of the GTP metabolic pathway was highly downregulated in the WT, most likely through direct interaction between RNA polymerase (RNAP) with (p)ppGpp (34). Our results indicate that a change in GTP level can indirectly affect the growth, viability, and virulence of *A. pleuropneumoniae*. Whether physiological characteristics could be affected by the GTP level was not identified in *E. coli*, but GTP is essential for survival of *B. subtilis* (38). In addition, we found that the promoter sequence features of the equivalent operon involved in this pathway in *A. pleuropneumoniae* were different from those in *E. coli* (34). More importantly, inhibition of the operon can be achieved at a basal level of (p)ppGpp. Future work will focus on cellular changes associated with above-basal levels of (p)ppGpp.

As in *B. subtilis*, changes in GTP levels were associated with growth effects in the (p)ppGpp^0 mutant. However, the purine regulatory mechanism appears to be different from that in *B. subtilis*, where the necessary 10 genes are in one operon (44). In *A. pleuropneumoniae* the 10 genes are instead spread throughout the genome. In *E. coli*, (p)ppGpp typically works in conjunction with DksA, a small transcription factor, to alter the transcription of many genes by binding to RNAP. Comparison of the amino acid sequences of DksA from *A. pleuropneumoniae* and *E. coli* determined that they share more than 64% identity. Additionally, the proposed two ppGpp binding sites of the RNAP of *E. coli* (45) are also conserved in the RNAP of *A. pleuropneumoniae*. Although another binding site of the RNAP region 2 β' subunit (R362) was replaced by H363 in *A. pleuropneumoniae* (data not shown), it seems likely that (p)ppGpp is binding RNAP/DksA to regulate transcription directly. In other bacteria, (p)ppGpp does not directly affect RNAP (4, 5). One possibility considered was whether *A. pleuropneumoniae* possesses a homologue of the global regulatory factor CodY found in Gram-positive bacteria (46–48). BLAST searches did not identify a CodY homologue or ortholog in the *A. pleuropneumoniae* genome. Despite nearly 5 decades of study, precisely how (p)ppGpp regulates cell growth remains poorly understood because the complete set of target proteins is unknown. Although some proteins have been reported to bind or be inhibited by ppGpp *in vitro* (42), the physiological relevance of these targets remains largely untested (with the exception of RNAP [49] and PurF [42] from *E. coli* and HprT [40] and GMK [41] from *B. subtilis*).

Similar to what is seen in *B. subtilis*, disruption of GTP homeostasis in an equivalent *B. subtilis* (p)ppGpp^0 mutant results in metabolic changes and decreased cell viability, even in the absence of starvation (38). Although we demonstrated that (p)ppGpp is associated with inhibition of transcription of enzymes involved in GTP biosynthesis in *A. pleuropneumoniae*, we were uncertain if this would lead to dysregulation of GTP homeostasis and changes in viability. So, to decrease or increase GTP levels transiently, the GMP synthetase (GuaA) inhibitor decoyinine or guanosine, which can be converted to GTP via the purine salvage pathway, was added to CDM. When the intracellular GTP level was reduced by addition of decoyinine, the growth of both the WT and (p)ppGpp^0 was decreased. The growth of (p)ppGpp^0 in stationary phase was elevated sharply by addition of guanosine, suggesting that GTP content was important for the growth and viability of *A. pleuropneumoniae*. Although the underlying mechanism of death caused by reducing GTP content is unclear, we demonstrated that a negative correlation between GTP and endogenous hydrogen peroxide content appears to exist and that this relationship seemed to depend on the basal level of (p)ppGpp.

Genome-wide effects on transcription from ppGpp in *E. coli* have shown that the basal level of ppGpp can downregulate the transcription of genes involved in BCAA biosynthesis and upregulate the transcription of genes involved in the purine biosynthesis pathway, while after accumulation of ppGpp beyond the basal level, the genes involved in these two pathways were regulated in opposite directions (34). This is

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consistent with the observation in *E. coli* that expression of the BCAA operons can be achieved by elevation of the basal ppGpp levels (32). In contrast, such regulation is possible with only a basal level of (p)ppGpp in *A. pleuropneumoniae*, with the transcription of genes involved in BCAAs and purine biosynthesis being upregulated and downregulated, respectively. This may result from the long-term adaptation of *A. pleuropneumoniae* to the BCAA-restricted environment of the lung, analogous to adaptation in humans of *Helicobacter pylori*, which has lost a large number of genes associated with *de novo* synthesis of purine nucleotides in the stomach niche (44).

Collectively, although we have a relatively good understanding of the underlying mechanisms by which (p)ppGpp modulates gene expression in the model organisms *B. subtilis and E. coli*, much less is known about the process in other species that ordinarily reside in different environmental niches. Our study indicates that *A. pleuropneumoniae* (p)ppGpp-mediated regulation differs from that of the model organism *E. coli* and is important for virulence, and further work will establish whether the results can be applied to other veterinary pathogens, especially those in the *Pasteurellaceae* family.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The bacterial strains, primers, and plasmids used in this study are described in Table S1 in the supplemental material. The *A. pleuropneumoniae* S-8 strain and mutant were cultured in tryptic soy agar (TSA) or tryptic soy broth (TSB) (Becton, Dickinson, Franklin Lakes, NJ) or CDM supplemented with 10 μg/ml NAD (50). The BCAAs were deleted from CDM when required. The Δ*relA*Δ $spoT_{App}$ double mutant [named the (p)ppGpp^0 mutant] was cultured in the presence of kanamycin (50 μg/ml) and erythromycin (5 μg/ml). *E. coli* β2155 (Δ*dapA*) was cultured in Difco Luria-Bertani (LB) broth (Miller) supplemented with 1 mM diaminopimelic acid (DAP) (Sigma-Aldrich, St. Louis, MO). Bacteria were treated with arginine hydroxamate (RHX; 0.5 mg/ml) to mimic amino acid starvation by depleting charged arginine tRNAs or with decoyinine (Dec; 50 μg/ml) to inhibit the GTP biosynthesis or with guanosine (Guo; 5 mM) to increase intracellular GTP pools via the salvage pathway. All bacteria were cultured at 37°C.

Construction of the (p)ppGpp^0 mutant. Deletion of the *relA* and *spoT* genes in *A. pleuropneumoniae* was performed as described previously (50, 51). First, the *relA* gene was replaced by a kanamycin resistance gene to construct the Δ$relA_{App}$ mutant, followed by replacement of the *spoT* gene with an erythromycin resistance gene. The final Δ*relA* Δ$spoT_{App}$ mutant was named the (p)ppGpp^0 mutant.

Detection of intracellular (p)ppGpp. To prove that the (p)ppGpp^0 mutant had lost the ability to synthesize (p)ppGpp, the WT and (p)ppGpp^0 mutant were streaked onto TSA plates, and a single colony was used to inoculate TSB and grown for 12 h. Subsequently, the culture was diluted to an optical density at 600 nm (OD_{600}) of 0.2 with fresh TSB and transferred to fresh TSB for an additional 2 h. Then, 1 ml of culture [WT and (p)ppGpp^0 mutant] was pelleted by centrifugation at 10,000 × *g* for 5 min, washed once with 1 ml of morpholinepropanesulfonic acid (MOPS), and resuspended in 250 μl of MOPS; [^{32}P]H_3PO_4 (Perkin Elmer) was added to 100 μCi/ml, and the culture was labeled for 1 h at 37°C. For the BCAA deprivation assay, the WT was cultured in CDM overnight and transferred to fresh CDM for an additional 2 h. After that, 1 ml of culture was pelleted and washed once with 1 ml of normal CDM; then the pelleted strains were resuspended in MOPS as a control, in CDM, and in CDM with deletion of a single BCAA and incubated with 100 μCi/ml [^{32}P]H_3PO_4 for another 1 h at 37°C. The intracellular (p)ppGpp was extracted and detected as performed previously (33, 52). Briefly, 50 μl of labeled culture was mixed with an equal volume of 2 M formic acid and placed on ice for at least 15 min. The mixture was centrifuged for 5 min at 16,000 × *g*, and 3 μl of the supernatant was spotted directly onto polyethyleneimine (PEI) cellulose thin-layer chromatography plates (Sigma), dried, and developed in 1.5 M KH_2PO_4 for 2.5 h. Nucleotides were visualized by autoradiography.

Growth experiments. The growth curve experiments were performed in CDM and CDM-BCAA. The initial optical density at 600 nm (OD_{600}) of all cultures was set at 0.02. Growth of three biological replicates for each strain was measured. The cultures were incubated at 37°C, aliquots were regularly withdrawn every 1 h, and the OD_{600} was measured using an Eppendorf spectrophotometer (Hamburg, Germany). All the growth curves are presented as $\log_2(OD_{600})$ versus time (53). The maximum growth rate (μ_{max}) was determined by comparing the slope (μ) of different adjacent time points. CFU counts were determined by counting serial dilutions on TSA plates that had been incubated overnight.

SEM. The pellets of the WT and the (p)ppGpp^0 mutant were harvested by centrifugation at 10,000 × *g* after growth for 12 h at 37°C, and scanning electron microscopy (SEM) was performed as described previously (33).

***Galleria mellonella* infection.** Assessment of the comparative virulence of WT and (p)ppGpp^0 mutant strains for *G. mellonella* larvae was performed as described previously (7). Briefly, groups of 10 larvae, ranging from 200 to 300 mg in weight, were randomly chosen and injected with 10-μl dilutions (1×10^6 to 1×10^9 CFU). Groups injected with phosphate-buffered saline (PBS) were used as negative controls. After injection, larvae were kept at 37°C, and survival was recorded at selected intervals. Experiments were performed independently three times with similar results.

RNA-seq analysis. The WT and (p)ppGpp^0 mutant were grown to log phase (4 h) in CDM and medium with deletions of single BCAAs. The cells were collected at 4°C and washed twice with precooled

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PBS buffer, and the pellets were frozen in liquid nitrogen. Subsequently, the samples were sent to the Beijing Genomics Institute (BGI)-Shenzhen in China (http://www.genomics.cn/index) in dry ice for sequencing. An RNeasy kit (Qiagen) and Ribo-Zero rRNA removal kit (Epicentre Biotechnologies) were used to isolate RNA and remove rRNA. A NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE) was used to quantify RNA. A total of 20 μg of RNAs for both the WT and (p)ppGpp^0 mutant was pooled for cDNA library construction. The cDNA libraries were constructed according to Illumina's protocols and sequenced using the Illumina HiSeq 2000 platform. This experiment was done on three biological replicates.

Differential expression analysis. The raw sequence reads were filtered using the Illumina pipeline. First, the reads with adaptor contamination and low quality or only one copy were excluded from the analysis. Then the remaining clean reads were mapped to the reference sequence of *A. pleuropneumoniae* S8 (GenBank accession no. ALYN00000000.1). The number of reads for each gene was determined and normalized between the libraries, and then the ratio of reads between the WT and (p)ppGpp^0 mutant was calculated. The differentially expressed genes which have a false discovery rate (FDR) threshold of 0.01 were identified as previously described (54, 55). The values of a $\log_2$ ratio of $\geq$1 and FDR of $\leq$0.001 were set as thresholds for significant differences in gene expression levels. Gene annotation was done according to the *A. pleuropneumoniae* S8 reference sequence (GenBank accession no. ALYN00000000.1) and *A. pleuropneumoniae* serovar 7 strain AP76 reference sequence (GenBank accession no. CP001091.1) using the blastn program (http://blast.ncbi.nlm.nih.gov/). The Gene Otology (GO) annotations were done by the Blast2GO program (http://www.geneontology.org). Pathway assignments were done according to the Kyoto Encyclopedia of Genes and Genomes pathway database (http://www.genome.jp/kegg).

Promoter sequence motif analysis. The homologous sequences and operons of the target genes involved in the purine biosynthesis pathway were searched by ProOpDB (56). The colinearity relationship between of *A. pleuropneumoniae* was analyzed by Mauve to get the operon distribution of target genes (http://darlinglab.org/mauve/mauve.html). The target genes were subjected to a BLAST search according to the reference genome sequence produced using Diamond to get the information about gene identification numbers (ID) and arrangement locations (57). Finally, promoter predictions were done according to the sequence which had been extracted from the 500 bp upstream of the first gene of the operon of the target genes (58).

H_2O_2 measurements. The production of H_2O_2 was measured using an H_2O_2-peroxidase assay kit (A22188; Life Technologies) (39). The WT and (p)ppGpp^0 mutant were grown to stationary phase (8 h) in different media, harvested by centrifugation at 4°C, and washed twice by assay buffer (50 mM Tris-HCl at pH 7.4), and the pellets were resuspended in the assay buffer. The final reaction mixture included equal volumes of cell suspension and reaction mix (20 μM Amplex UltraRed and 0.2 U ml^{-1} horseradish peroxidase) in 96-well microtiter plates at 37°C for 30 min. The absorbance was read at 560 nm by a BioTek ELx808 spectrophotometer. To normalize absorbance by CFU count, the cell aliquots from different cultures were serially diluted and plated onto TSA for colony counting.

Statistical analysis. All statistical analyses were conducted with the GraphPad Prism, version 5, software. All data were collected from three biological replicates and are expressed as the arithmetic means $\pm$ standard deviations (SD).

Data availability. The raw transcriptome analysis (RNA-seq) data have been deposited in ArrayExpress (EMBL-EBI [https://www.ebi.ac.uk/arrayexpress/]) and are accessible through accession number E-MTAB-8514.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 2.1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.03 MB.

ACKNOWLEDGMENTS

This work was funded by the National Natural Science Foundation of China (grants 31672575 and 31772757) and the Heilongjiang Province Natural Science Foundation (grant C2017078). Paul R. Langford was supported by the UK Biotechnology and Biological Sciences Research Council (BB/S019901/1 and BB/K020765/1).

We thank Zhai Lei (China National Research Institute of Food and Fermentation Industries) for technical assistance with the SEM experiments.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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