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Co-regulation of CodY and (p)ppGpp synthetases on morphology and pathogenesis of *Streptococcus suis*

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ABSTRACT

CodY and (p)ppGpp synthetases are two important global regulators of bacteria. In some pathogens, such as *Listeria monocytogenes*, the GTP pool links these two regulatory systems, and introducing a *codY* mutant into the Δ*relA* strain restored the pathogenicity of the attenuated Δ*relA* mutant. In previous studies, we identified the (p)ppGpp synthetases (RelA and RelQ) and CodY of *Streptococcus suis*. To understand the interrelationships between these two regulators in *S. suis*, a Δ*relA*Δ*relQ*Δ*codY* mutant was constructed, and its growth, morphology, and pathogenicity were evaluated. Compared with Δ*relA*Δ*relQ*, Δ*codY*, its growth was very slow, but its chain length was partly restored to the wild-type length and its capsule became thick and rough. The adherence, invasion ability, and resistance to whole-blood killing *in vitro* of Δ*relA*Δ*relQ*Δ*codY* and its lethality and colonization ability in mice were clearly reduced, which differs from the effects of these mutations in *L. monocytogenes*. An analysis of gene expression showed that CodY interacted with the *relA* promoter in a GTP-independent manner to positively regulate the expression of *relA*. The introduction of a *codY* mutant into the Δ*relA*Δ*relQ* strain further reduced the expression of virulence factors, which suggests a novel interaction between the (p)ppGpp synthetases and CodY. This study extends our understanding of the relationship between the (p)ppGpp-mediated stringent response and the regulation of CodY in *S. suis*.

1. Introduction

During the processes of survival and infection, bacterial pathogens are exposed to various environmental challenges, and one such unfavorable factor is nutrient insufficiency. Several regulators contribute to bacterial nutrient sensing, including two-component systems, CcpA, Rel/SpoT homologues (RSH), and CodY (Willenborg et al., 2014; Zhang et al., 2016; Zheng et al., 2018). Among these, RSH and CodY are two important regulators of *S. suis* (Feng et al., 2016; Zhu et al., 2016). RSH is a guanosine pentaphosphate or tetraphosphate ([p]ppGpp) synthetase, which controls the pool of stringent response signal molecules ([p]ppGpp) and can reprogram transcription during amino acid starvation (Potrykus and Cashel, 2008; Traxler et al., 2008). CodY is a DNA-binding repressor that regulates the biosynthesis of branched-chain amino acids (BCAA) (Li et al., 2013a; Kim and Burne, 2017).

In *Escherichia coli* and some other Gram-negative bacteria, two RSH enzymes (RelA and SpoT) are involved in (p)ppGpp synthesis. The first enzyme RelA is recognized to respond to amino acid starvation, and another homologous enzyme SpoT senses many other environmental stresses, such as starvation of carbon, iron, phosphate and fatty acids (Traxler et al., 2008). In contrast, Gram-negative α-Proteobacteria and most Gram-positive bacteria contain a single RSH, which respond to not only amino acid starvation but also other stresses, such as Rel_{Bsu} in *Bacillus subtilis*, RelA in *Listeria monocytogenes*, and Rel_{Smu} in *Streptococcus mutans* (Primm et al., 2000; Bennett et al., 2007; Lemos et al., 2007). In adddtion, RelP, RelQ and some small alarmone synthases

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(SASs) were also reported in some bacteria. The accumulation of (p)ppGpp affects not only gene expression and metabolism, but also controls various virulence-related physiological phenotypes in pathogens, including the expression of pneumolysin toxin and other virulence factors in *S. pneumoniae* (Kazmierczak et al., 2009), the long-term persistence of *Mycobacterium tuberculosis* (Dahl et al., 2003), biofilm formation by *L. monocytogenes* (Renier et al., 2011), and the survival of *Staphylococcus aureus* after phagocytosis (Geiger et al., 2012). The virulence of most RSH-deficient pathogens is attenuated (Dahl et al., 2003; Kim and Burne, 2017).

CodY was first described in *B. subtilis*, where it functions as a repressor of the *dppABCDE* operon by binding to its promoter DNA (Fisher et al., 1996; Bergara et al., 2003). It was then identified in many low-(G + C) Gram-positive bacteria (Geiger and Wolz, 2014). The affinity of CodY for its target DNA is enhanced when it is bound to GTP and/or BCAA (Guedon et al., 2001). As a nutrient regulator, further studies showed that many virulence factors are expressed in a CodY-dependent manner in some pathogens, including the anthrax toxins of *Bacillus anthracis* and the hemolysins and capsular polysaccharides of *Staphylococcus aureus* (Majerczyk et al., 2008; Willem et al., 2009).

CodY and (p)ppGpp regulate the expression of many common phenotypes, and even some genes, suggesting their potential coordinated regulation (Geiger et al., 2010). In recent reports, GTP levels have been shown to link the (p)ppGpp-mediated stringent response and the regulation of CodY (Inaoka and Ochi, 2002; Geiger et al., 2010). In *L. monocytogenes*, the *ΔrelA* mutant is attenuated, the expression of its CodY regulon is reduced in the early stationary phase, and the introduction of a *codY* mutant into the Δ*relA* strain restored its virulence (Bennett et al., 2007). This explanation was as follows. RSH-dependent (p)ppGpp accumulation reduced the GTP pool, and GTP is the ligand of CodY. In contrast, in strain Δ*relA*, the high level of GTP continued to repress the CodY regulon until the expression of CodY was knocked out (Geiger and Wolz, 2014). The virulence of an attenuated Δ*rsh* mutant of *S. aureus* was also restored by the additional mutation of *codY* (Geiger et al., 2012), which could also be attributed to the (p)ppGpp-induced derepression of some CodY regulon. However, recent studies have shown that GTP is not a cofactor of CodY in some streptococci, including *S. pyogenes* and *S. mutans* (Malke et al., 2006; Lemos et al., 2008), so the link between the stringent response and the regulation of CodY is still unclear in these bacteria.

Streptococcus suis is a Gram-positive swine pathogen and an important zoonotic agent that causes meningitis, arthritis, septicemia, and endocarditis (Yu et al., 2006). As an emerging zoonotic pathogen, *S. suis* serotype 2 has become the predominant causative agent of adult human meningitis in Vietnam and Hong Kong (Wertheim et al., 2009; Weinert et al., 2015). In previous studies, we identified the (p)ppGpp synthetases and CodY of *S. suis*, and demonstrated their roles in the global regulation of the bacterium (Feng et al., 2016). The virulence of both the (p)ppGpp synthetase mutant (*ΔrelAΔrelQ*) and the *codY* mutant (Δ*codY*) was attenuated (Feng et al., 2016; Zhu et al., 2016). In this study, in order to further investigate the co-regulation of CodY and (p)ppGpp synthetases on morphology and pathogenesis of *S. suis*, we characterized the growth, virulence, and related gene expression of the *ΔrelAΔrelQΔcodY* mutant and identified the link between the (p)ppGpp synthetases and CodY in streptococci, using the *S. suis* model.

2. Materials and methods

2.1. Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are shown in Table 1. The parental strain SC-19 was isolated from a diseased pig in Sichuan Province of China in 2005 (Yu et al., 2006). SC-19 and its genetically modified strains were grown in tryptic soy broth (TSB; Difco, Illkirch, France) or plated on tryptic soy agar (TSA; Difco) containing 5% (vol/vol) newborn bovine serum (Sijiqing, Hangzhou, China) at 37 °C. The *Escherichia coli* strains used for gene cloning and protein expression were cultured in Luria–Bertani (LB) broth (Difco) or plated on LB agar plates at 37 °C or 18 °C. To count colony-forming units (CFU), bacterial cultures were vortexed for 10 min to break the long bacterial chains, and were then plated onto TSA.

Table 1
S. suis strains and plasmids used in this study.

Strains and plasmids	Description	Reference or source
Bacterial strains		
SC-19	Wild type	Lab collection
ΔrelAΔrelQ	*relA*::erm *relQ*	Lab collection (Zhu et al., 2016)
Δ*codY*	*codY*::spc	Lab collection (Zhu et al., 2016)
ΔrelAΔrelQΔcodY	*relA*::erm *relQ codY*	This study
E. coli DH5α	Cloning recombinant plasmid	Promega
Plasmids		
pSET4s	*E. Coli-S. suis* shuttle vector; Spc^r	(Takamatsu et al., 2001b)
pSET4sΔ*codY*	A recombinant vector, designed for knockout of *codY* from *ΔrelAΔrelQ*	This study

2.2. Construction of the ΔrelAΔrelQΔcodY mutant

All the primers were designed based on the genomic sequence of *S. suis* 05ZYH33 (GenBank accession no. CP000407) and are listed in Table 2. Two pairs of specific primers (YL-1/2 and YR-1/2) were used to clone the up- and down-stream homologous regions *codY*, respectively. The PCR product was cloned into the pSET4s vector to create pSET4s::*ΔcodY*, which was confirmed by DNA sequencing. *ΔrelAΔrelQ* strain cells were then transformed with pSET4s::*ΔcodY* and the *ΔrelAΔrelQΔcodY* mutant was selected as previously reported (Takamatsu et al., 2001). The suspected mutant was confirmed by PCR and reverse transcription PCR (RT-PCR). The expression of the genes up- and downstream from *codY* was also confirmed by qPCR.

2.3. Microscopic imaging

Gram staining was performed as described previously (Zhu et al., 2016). The stained samples were observed with light microscopy. Scanning electron microscopy (SEM) was performed as in a previous study (Wang et al., 2011), with some modifications. Cells in mid-log phase (with an optical density at a wavelength of 600 nm [OD_{600}] of 0.6) were collected and placed on glass coverslips (0.17 mm thick and 20 mm in diameter; WHB, Shanghai, China). The samples were fixed overnight with 2.5% glutaraldehyde at 4 °C and then dehydrated in increasing concentrations of ethanol (30%, 50%, 70%, 90%, and twice in 100%) for 15 min each. After the samples were dried, the glass coverslips were stuck onto conductive glue, and sprayed with gold with an ion sputtering instrument (JSM-6390LV, JEOL, Tokyo, Japan) and observed with SEM (JFC-1600, JEOL, Tokyo, Japan). Ten chains of SC-19 cells or the *ΔrelAΔrelQ* strain cells were randomly selected from the images and measured. Transmission electron microscopy (TEM) was performed as described previously (Zheng et al., 2011). Cells in mid-log phase (OD_{600} 0.6) were harvested and washed twice with phosphate-buffered saline (PBS). The cells were then fixed overnight in 2.5% glutaraldehyde at 4 °C. After dehydration in alcohol, the samples were embedded and cut into thin slices to be observed with TEM (H-7650, Hitachi Ltd. Tokyo, Japan). The method has been described in a previous study (Hammerschmidt et al., 2005; Fujita et al., 2007). Ten capsules of each strain were randomly selected from the images and measured.

Table 2
The used primers in this study.

Primers	Sequence	Gene locus / Functions
Neg-1	ACCGTTACTAAATTTTTATCTTGC	EMSA
Neg-2	TGCTAAACATCTCCTTAGTTTTAT	
EcodY-1	TAGATAATGGGAGTGGGAAA	EMSA
EcodY-2	CAGTATAGCAAAAAATACATAAAAT	
ErelA-1	CATATCTCTACTCTTCCTC	EMSA
ErelA-2	AGCTAGTGTGAGTGCTAC	
His-Y-1	CGTACGGGATCCATGACAACATTATTAGAGAAG	Expression of His-CodY protein
His-Y-2	CGCTACCTCGAGTTAGTAGTCACGTTTCTTAATT	
YL-1	CGTACGCTGCAGCCAGATTTATTTGTTGTGACGA	Construct the left arm of pSET4s::*codY*
YL-2	CGCTACGTCGACATTTTCCTCTTTACTCTGTAAT	
YR-1	CGTACGGGATCCACTTATTTGAAAGTTCTCATC	Construct the right arm of pSET4s::*codY*
YR-2	CGCTACGAATTCGGTAGTACAATTTAGCACGACG	
Y-1	TGCGGCAATATCGTAGATAA	Detection Δ*relA*Δ*relQ*Δ*codY* by PCR
Y-2	GAGCTTCAGGATGAAATTCA	
350-1	TTGAAGCGTTCCGAAGAGC	Detection Δ*relA*Δ*relQ*Δ*codY* by RT-PCR
350-2	TGTAATACCGATACGATCTGC	
349-1	GTGACTTGATTCATAACGCC	Detection Δ*relA*Δ*relQ*Δ*codY* by RT-PCR
349-2	GGAGCCAGTGTCGCAATAG	
351-1	GTGGCAGATGAAGGAAAGCTG	Detection Δ*relA*Δ*relQ*Δ*codY* by RT-PCR
351-2	TCAATGCAGCAGTGGCTGAT	
mrp-1	GGGTATGATTATGTGGCAACTA	SSU05_0753 / For qRT-PCR (Zhu et al., 2016)
mrp-2	TGTAAGCAACTCAATAAACCCA	
cps2A-1	TATTGGCTGGATTATTGATGTG	SSU05_0564 / For qRT-PCR (Zhu et al., 2016)
cps2A-2	CTGGGACAAGGATACTCATTTC	
cps2B-1	ATCAAGAAGTAAATGCAATCG	SSU05_0565 / For qRT-PCR (Zhu et al., 2016)
cps2B-2	CTACATAGATACGGGTAG	
cps2E-1	CACATTAATAAACTTCGTTTTG	SSU05_0568 / For qRT-PCR (Zhu et al., 2016)
cps2E-2	CTTGCATATTTTCCCATAGT	
cps2F-1	CTATTGAAATTGCCAAAGATA	SSU05_0569 / For qRT-PC (Zhu et al., 2016)
cps2F-2	CGTAGCCATTCATGACCG	
cps2H-1	ATAATACCTTTGGATGCGGA	SSU05_0571 / For qRT-PCR
cps2H-2	TCAGAATGATGCCAAACAGG	
cps2J-1	ATTTGTCGGGAGGGTTACTT	SSU05_0573 / For qRT-PCR (Zhu et al., 2016)
cps2J-2	AAGTTTGCAACAAGGGCTAT	
arcA-1	TCTTCGATGATATCCCATTCTT	SSU05_0624 / For qRT-PCR (Zhu et al., 2016)
arcA-2	GCTTACGAATTGCCTTTTTAGT	
sod-1	GGCAGCAGATATTGATGC	SSU05_1539 / For qRT-PCR (Zhu et al., 2016)
sod-2	AGATAACTTCCAACTTGCC	
gapdH-1	CTTGGTAATCCCAGAATTGAACGG	SSU05_0155 / For qRT-PCR (Zhu et al., 2016)
gapdH-2	TCATAGCAGCGTTTACTTCTTCAGC	
fbps-1	GGTGGCCCAGCAGGCCAATG	SSU05_1492 / For qRT-PCR (Zhu et al., 2016)
fbps-2	CCGCCAATTCCTGCTCCTGC	
epf-1	CTTACTTAGATGGTCCAAATGGGTCA	SSU05_0177 / For qRT-PCR (Zhu et al., 2016)
epf-2	AACTTATCAAAAATAGCCTGTTCCG	
eno-1	CGTGATCAACAAGCTATC	SSU05_1503 / For qRT-PCR (Zhu et al., 2016)
eno-2	GTGTAAAGTGGCACTTCAA	
sao-1	TTTGGTCGTGAGCTTCTAG	SSU05_1371 / For qRT-PCR
sao-2	CCAGGCTTATCGAATGATAG	
gpsB-1	AAGTGAACGATTTTCTTGATGAT	SSU05_0417 / For qRT-PCR (Zhu et al., 2016)
gpsB-2	GATGGACGTTCTGTACGCAAT	
divIVA-1	AAGGTTGCGGCTGAAGAT	SSU05_0487 / For qRT-PCR
divIVA-2	TGATTTTAGACGCTGATGGA	
relA-1	AATCTATGCACCGCTTGCCCAC	SSU05_2094 / For qRT-PCR
relA-2	TGTATAATCTCTTAGCTTCTCA	
16S rRNA-1	GTAGTCCACGCCGTAAACG	SSU05_2255 / For qRT-PCR (Zhu et al., 2016)
16S rRNA-2	TAAACCACATGCTCCACCGC	

*RS: restriction sites. Underline letters indicated restriction sites.

2.4. Cell adhesion and invasion assays

The adherence and invasion assays were performed as described previously (Li et al., 2013b), with some modifications. Human epidermoid cancer cells (HEp-2) were cultured in Dulbecco's modified Eagle's medium (Hyclone, Utah, USA) and grown in 75-cm^2 flasks at 37 °C under a 5% CO_2 humidified atmosphere. Cells from a confluent monolayer (1.0×10^4 cells) were then cultured in each well of a 12-well plate (Corning, NewYork, USA). SC-19, *ΔrelAΔrelQ*, *ΔcodY*, or *ΔrelAΔrelQΔcodY* cells were then added to individual wells (multiplicity of infection [MOI] = 100:1) and incubated at 37 °C to allow the bacteria to attach to the cells. After incubation for 2 h, all the plates were washed twice with PBS and the cells were lysed with sterile distilled water on ice. Both the adherent and intracellular bacteria were counted on TSA. To count the invasive bacteria, ampicillin (100 μg/ml) was added to the HEp-2 medium and the plates were incubated for a further 2 h. The cells were lysed with sterile distilled water on ice and the bacteria were counted on TSA. Each assay was carried out at least three biological replicates.

2.5. Whole-blood bactericidal assay

Whole blood bacterial assay was performed as described previously (Liu et al., 2004), with some modifications. Strain SC-19 and all the mutant strains were cultured to early stationary growth phase at 37 °C. Approximately 1×10^4 CFU bacteria (100 μl) were mixed with 900 μl

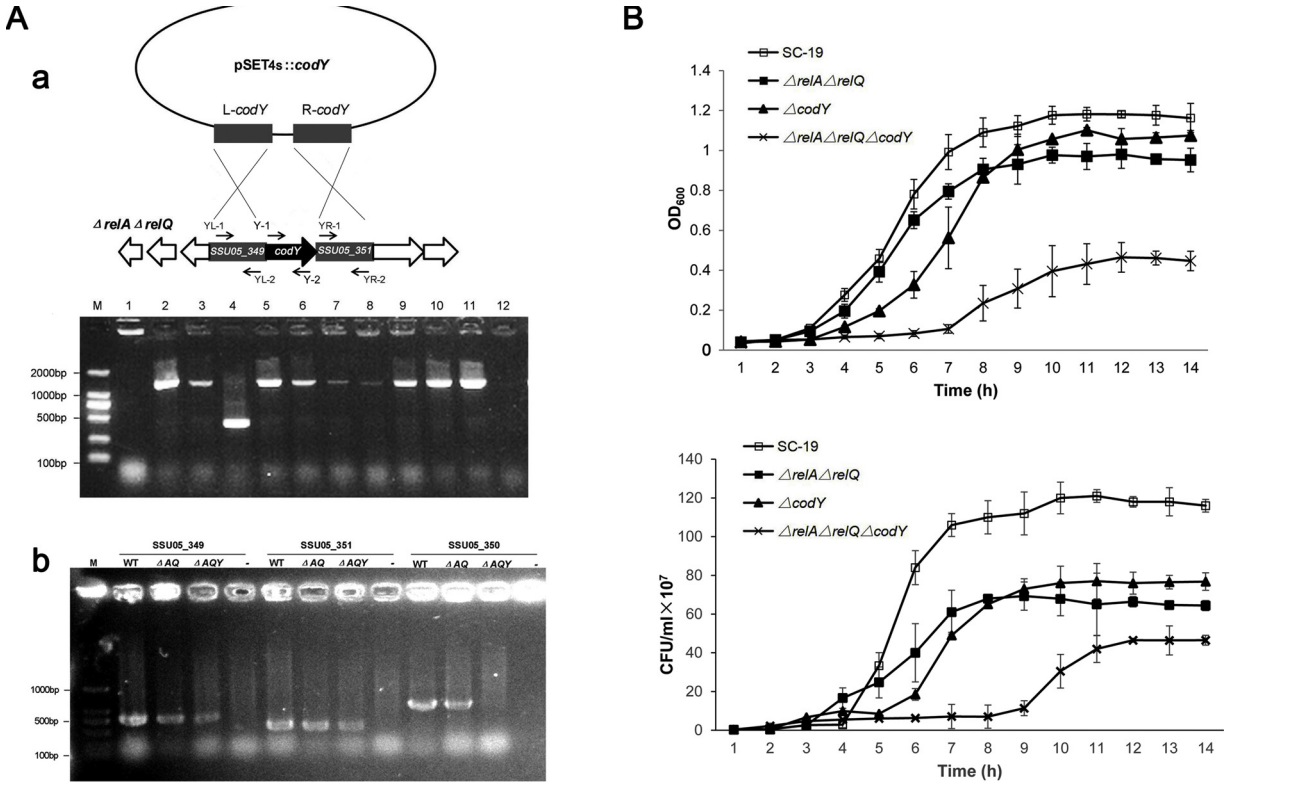


Fig. 1. Construction of Δ*relA*Δ*relQ*Δ*codY* mutant and the growth curves of *S. suis* strain SC-19. (A-a) To evaluate the role of CodY to Δ*relA*Δ*relQ*, the entire *codY* gene was deleted in Δ*relA*Δ*relQ* strain by using Homologous recombination. To make the deletion, we construct pSET4$_S$-*codY*RF recombinant plasmid to acquire the Δ*relA*Δ*relQ*Δ*codY*. The primers YL-1/2, YR-1/2 and Y-1/2 which were used for constructing and detecting the *codY* mutant were shown in Table 2. The sequence of the mutant which had knocked out *codY* was 675 bp while the WT or reverting mutant strain was 1404 bp. (A-b) The expression of upstream gene (SSU05_0349) and downstream gene (SSU05_0351) of *codY* (SSU05_0350) was detected by primer 349-1/2 and 351-1/2 respectively of each strain by RT-PCR. (B) Growth curves of SC-19 (□), Δ*relA*Δ*relQ* (■), Δ*codY* (▲) and Δ*relA*Δ*relQ*Δ*codY* (×) strains in TSB. Bacteria cell density was measured spectrometrically at OD_{600}. The bacterial numbers of SC-19 (□), Δ*relA*Δ*relQ* (■), Δ*codY* (▲) and Δ*relA*Δ*relQ*Δ*codY* (×) strains on TSA. The CFU number was counted per hour. All data were shown as means ± SD from three replicates.

of whole mouse blood and incubated for 2 h with shaking at 160 rpm. The diluted blood samples (100 µl) were then plated on TSA to count the surviving bacteria. Each assay was carried out with at least three biological replicate.

2.6. Mouse infection assay

The pathogenicity of *S. suis* was further examined in specific-pathogen-free (SPF) Kun-Ming mice as the infection model. All mice used in this study were purchased from the Wuhan Institute of Biological Products (Wuhan, China) under license number SYXK (E) 2010-0029. The mouse infection experiments were approved by the Ethics Committee of Huazhong Agricultural University and were performed according to the Hubei Province Laboratory Animal Management Regulations 2005. All efforts were made to minimize the animals' suffering. The female Kun-Ming mice (6-weeks-old) were divided into five groups (six mice per group) to measure the pathogenesis of SC-19, Δ*relA*Δ*relQ*, Δ*codY*, and Δ*relA*Δ*relQ*Δ*codY*. Approximately 8×10^8 CFU/ml of each strain in 500 µl of PBS was injected into the mouse peritoneum. The mortality of the mice infected with the different bacterial strains was recorded daily over a 7-d period. PBS was injected as the negative control.

To detect viable bacteria in the mouse organs, 120 6-week-old female Kun-Ming mice were divided into four groups and injected with SC-19, Δ*relA*Δ*relQ*, Δ*codY*, or Δ*relA*Δ*relQ*Δ*codY* at an approximate dose of 5×10^7 CFU/ml. After 18, 24, 48, 72, 96, and 168 h post injection, five infected mice from each group were killed by cervical dislocation and their blood and tissues harvested. The numbers of viable bacteria in the blood, brain, spleen, and lungs were counted by plating the tissue extracts on TSA.

2.7. Electrophoretic mobility shift assay (EMSA)

The coding sequence of *codY* was amplified from the genomic DNA of *S. suis* SC-19 and cloned into the prokaryotic expression vector pET-28a (Novagen, Shanghai, China). *Escherichia coli* BL21(DE3) cells were transformed with the recombinant plasmid pET28a-*codY* and expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 18 °C for 12 h. The recombinant proteins were purified with Ni-NTA agarose (Bio-Rad, Shanghai, China) under native conditions, according to the manufacturer's instructions.

To detect the affinity of CodY for DNA, DNA fragments of the candidate promoters were amplified and purified with the PCR Product Purification Kit (Sangon Biotech, Shanghai, China). The binding reactions were performed in a 20 µl volume containing the binding buffer (20 mM Tris-HCl [pH 8.0], 50 mM KCl, 5% (v/v) glycerol, 0.5 mM dithiothreitol, 25 µg/ml bovine serum albumin, 100 ng poly[dI–dC]), 0.1 µg promoter DNA, and different concentrations of the purified recombinant CodY protein (0, 50, 100, 200, or 400 nM). The reaction mixtures were incubated at 37 °C for 15 min. Loading buffer was then added and electrophoresis was performed on 5% (w/v) native polyacrylamide DNA-retardation gels at 100 V for 1 h. The gels were stained with ethidium bromide. The 214-bp promoter of SSU05_1076 was used as the negative control. The 387-bp promoter of *codY* was used as the positive control (Feng et al., 2016). To detect the effect of GTP on CodY binding, different concentrations (0, 3, 6, 12, or 24 mM) of GTP (Sigma,

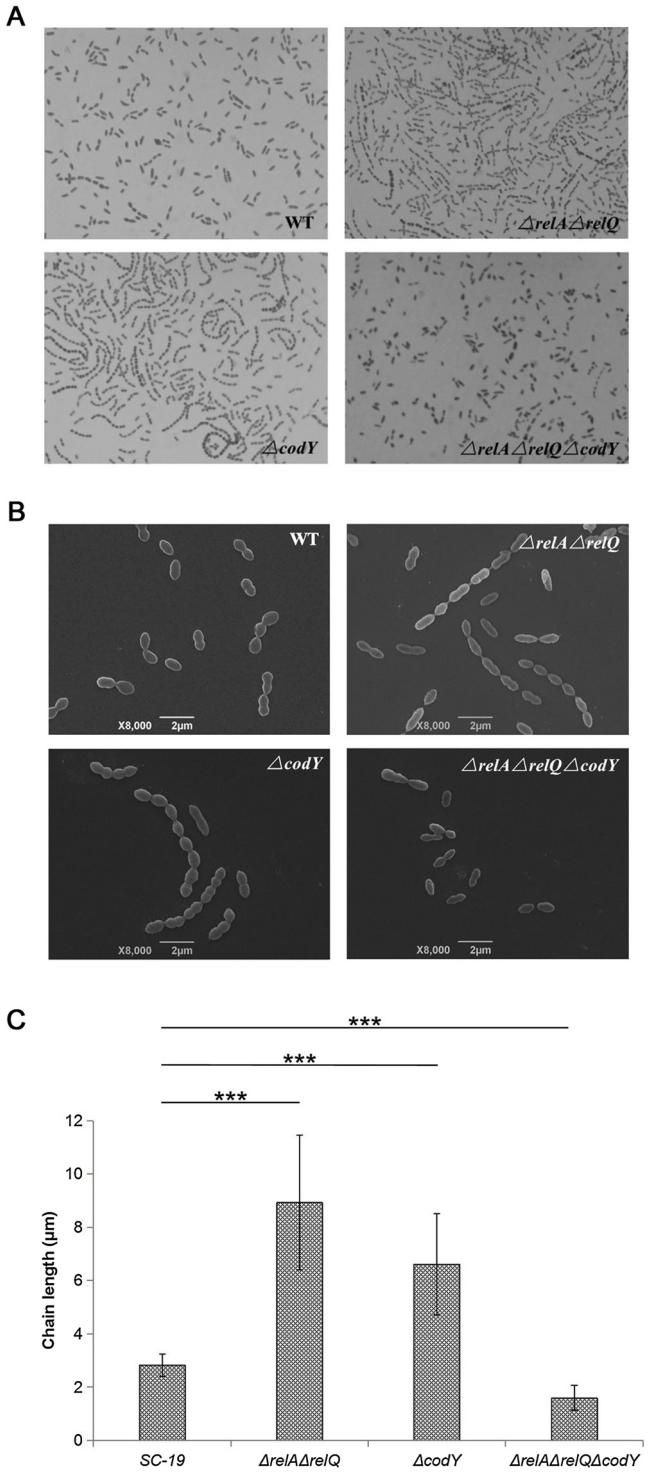


Fig. 2. Chain length and morphology of SC-19, Δ*relA*Δ*relQ*, Δ*codY* and Δ*relA*Δ*relQ*Δ*codY* strains. (A) Light microscope morphology of *S. suis* strains was observed by Gram staining (×1000). (B) Chain Length among *S. suis* strains was observed by Scanning Electron Microscope (×8000). The bar was 2 μm. (C) Average chain-length of each strain. Data were shown as means ± SD. (***$p < 0.001$).

USA) were added to the reaction mixtures.

2.8. Quantitative reverse transcription PCR (qRT-PCR)

The strains SC-19, Δ*relA*Δ*relQ*, Δ*codY*, and Δ*relA*Δ*relQ*Δ*codY* were grown overnight at 37 °C in TSB with 5% newborn bovine serum, and then diluted 100-fold with fresh medium. After further incubation for 6 h, the cells in the exponential growth period were collected. Their total RNA was isolated and purified with the Qiagen RNeasy Mini Kit (Qiagen, Shanghai, China), according to the manufacturer's instructions. Contaminating DNA was removed by treating the samples with RNase-free DNase I. cDNA was synthesized with the PrimeScript RT Reagent Kit (TaKaRa, Dalian, China), according to the manufacturer's instructions. qRT-PCR was performed on an ABI 7500 H T Sequence Detection System (Applied Biosystems, Forster, CA, USA) using SYBR Green Master Mix (Vazyme, Nanjing, China). The expression level of each gene was calculated with the comparative cycle threshold formula ($2^{-\Delta\Delta Ct}$) and normalized to the 16S rRNA level (Livak and Schmittgen, 2001). The primers were designed based on the *S. suis* 05ZYH33 genome sequence (Table 2).

2.9. Statistical analysis

All data were used ANOVA to analyze. The data between different mutants and WT were analyzed with Student's *t*-test. Differences were considered significant at a p value < 0.05 (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). $p > 0.05$ was regarded as not significant (ns).

3. Results

3.1. Construction of the ΔrelAΔrelQΔcodY mutant

As shown in Fig. 1A, the Δ*relA*Δ*relQ*Δ*codY* mutant was constructed from strain Δ*relA*Δ*relQ* using a homologous recombination method, and confirmed with PCR and RT-PCR. The expression of SSU05_0349 and SSU05_0351 was also confirmed, and suggested that the deletion of *codY* did not affect the transcription of its neighboring genes.

3.2. Effects of (p)ppGpp synthetases and CodY on normal growth of S. suis

In TSB medium, the growth of the Δ*relA*Δ*relQ* and Δ*codY* mutants was slightly slower than that of SC-19, whereas the Δ*relA*Δ*relQ*Δ*codY* mutant displayed the slowest growth of all the strains. The bacterial numbers of each strain were consistent with the OD_{600} values (Fig. 1B). These results indicate that the (p)ppGpp synthetases and CodY are essential for maintaining the normal growth of *S. suis*.

3.3. Effects of (p)ppGpp synthetases and CodY on bacterial cell morphology

To investigate the influence of CodY and the (p)ppGpp synthetases on the cell shape, chain length, and capsular structure of *S. suis*, all the strains were observed under light microscopy and/or electron microscopy. As shown in Fig. 2, the chain lengths of the Δ*relA*Δ*relQ* and Δ*codY* mutants were significantly longer than that of SC-19 ($p < 0.001$), whereas that of Δ*relA*Δ*relQ*Δ*codY* was significantly shorter than that of SC-19 ($p < 0.001$). The capsule of mutant Δ*codY* was thinner than that of SC-19 ($p < 0.05$), but the capsule thicknesses of Δ*relA*Δ*relQ* and SC-19 did not differ significantly ($p > 0.05$). However, the capsule of Δ*relA*Δ*relQ*Δ*codY* was significantly thicker than those of the WT and other mutants ($p < 0.001$), and the surface of the capsule was rough while that of the other mutants were smooth (Fig. 3A). The shapes of Δ*relA*Δ*relQ* and Δ*codY* cells were spheres or elliptical spheres, and the cells divided normally, like the parental strain. However, the Δ*relA*Δ*relQ*Δ*codY* mutant appeared as non-homogeneous spheres and displayed asymmetric cell division (Fig. 3B). Most Δ*relA*Δ*relQ*Δ*codY* cocci were malformed and irregular, suggesting that the deletion of the (p)ppGpp synthetases and CodY caused immature cell development.

3.4. Effects of (p)ppGpp synthetases and CodY on adhesion, invasion, and resistance to whole-blood killing in vitro

The adhesion and invasion abilities of each strain were evaluated in

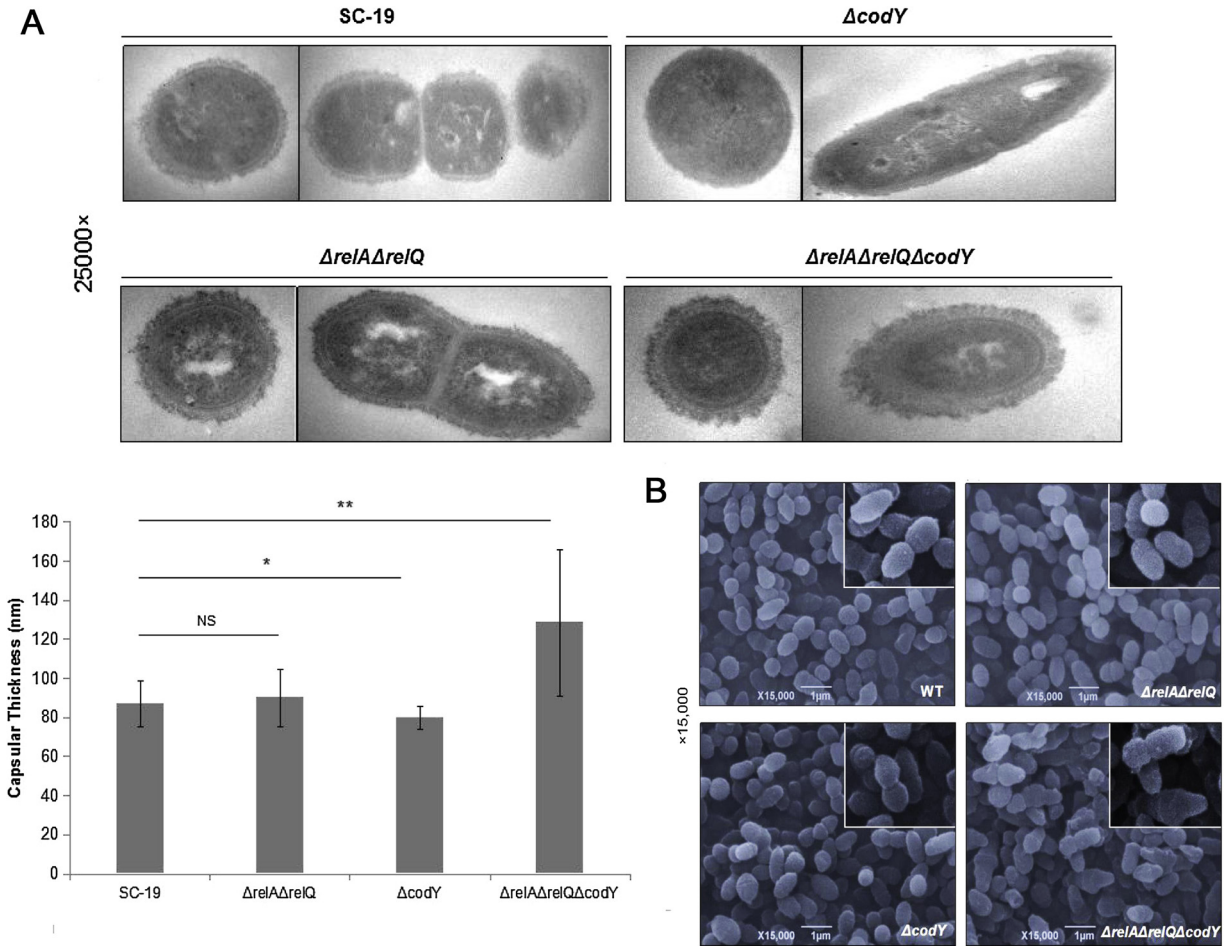


Fig. 3. Capsular thickness of SC-19, Δ*relA*Δ*relQ*, Δ*codY* and Δ*relA*Δ*relQ*Δ*codY* strains. (A) Transmission electron microscopy was used to detect the capsular of SC-19, Δ*relA*Δ*relQ*, Δ*codY* and Δ*relA*Δ*relQ*Δ*codY* strains (×25,000). Data are shown as means ± SD. The bar was 200 nm. (B) Morphology of SC-19, Δ*relA*Δ*relQ*, Δ*codY* and Δ*relA*Δ*relQ*Δ*codY* strains was observed by Scanning Electron Microscope (×15,000). The bar was 1 μm. (NS was no significant, *$p < 0.05$, **$p < 0.01$).

HEp-2 cells. As shown in Fig. 4A and B, the adhesion and invasion abilities of Δ*relA*Δ*relQ*, Δ*codY*, and Δ*relA*Δ*relQ*Δ*codY* were significantly reduced ($p < 0.001$). Among these strains, the Δ*relA*Δ*relQ*Δ*codY* mutant showed the lowest adhesion and invasion rates. In the whole-blood killing assay, the numbers of surviving mutant bacteria were lower than the number of surviving SC-19 bacteria (Fig. 4C), and Δ*relA*Δ*relQ*Δ*codY* showed the lowest survival rate. These results suggest that the inactivation of the (p)ppGpp synthetases and/or CodY reduced the resistance of *S. suis* to whole-blood killing.

3.5. Effects of (p)ppGpp synthetases and CodY on virulence and colonization

The virulence of each mutant was evaluated in an SPF mouse infection model. As shown in Fig. 5A, all SPF mice inoculated with SC-19 died within 24 h of infection, whereas 33.3% of the mice infected with Δ*codY* survived. In contrast, all the mice infected with Δ*relA*Δ*relQ* and Δ*relA*Δ*relQ*Δ*codY* survived. Colonization assays were also performed. As shown in Fig. 5B, Δ*relA*Δ*relQ* and Δ*relA*Δ*relQ*Δ*codY* had been cleared from the blood and brain at 96 h post-infection and from the spleen and lung at 168 h post-infection, whereas Δ*codY* had been cleared from the lung at 168 h post-infection, but at which point it was still detectable in the blood, brain, and spleen. In contrast, SC-19 was still present in all the samples at 168 h post-infection. These results suggest that CodY and especially the (p)ppGpp synthetases are essential for maintaining the virulence.

3.6. Expression of genes related to morphology and pathogenesis

To explain the phenotypes described above, 16 genes were analyzed with qRT-PCR. Of these genes, *gapdH*, *fbps*, *eno*, *cps*, *arcA*, *mrp*, *sod*, *epf*, and *sod* encode important virulence factors, and *cps*, *divIVA*, and *gpsB* encode proteins involved in cell morphology. As shown in Fig. 6, the expression of three genes (*cps2J*, *mrp*, and *sao*) was up-regulated and that of six genes (*gapdH*, *fbps*, *gpsB*, *arcA*, *sod*, and *epf*) was down-regulated in the Δ*relA*Δ*relQ* mutant relative to SC-19. Three genes (*gapdH*, *gpsB*, and *sod*) were up-regulated and six genes (*fbps*, *cps2H*, *cps2J*, *arcA*, *mrp*, and *sao*) were down-regulated in the Δ*codY* mutant relative to SC-19. In the Δ*relA*Δ*relQ*Δ*codY* mutant, two genes (*cps2H* and *divIVA*) were up-regulated and 10 genes (*gapdH*, *fbps*, *eno*, *cps2A*, *cps2J*, *gpsB*, *arcA*, *mrp*, *sao*, and *epf*) were down-regulated relative to SC-19.

3.7. CodY interacts with the relA promoter in a GTP-independent manner

The recombinant CodY protein was purified and the relevant promoters were amplified with PCR (Fig. 7A and B). As shown in Fig. 7C, the migration of the *relA* promoter DNA and the *codY* promoter DNA was retarded when CodY was added. In contrast, no shift was detected in the negative control. To determine whether GTP is a co-effector of CodY, we tested the binding of CodY to the *relA* promoter in the presence of GTP. When 6 mM GTP was added to the binding reaction mixture, the binding ability of CodY was not increased (Fig. 7E). Similarly, the binding ability of CodY was not increased when the concentration of GTP was increased. We also compared the expression

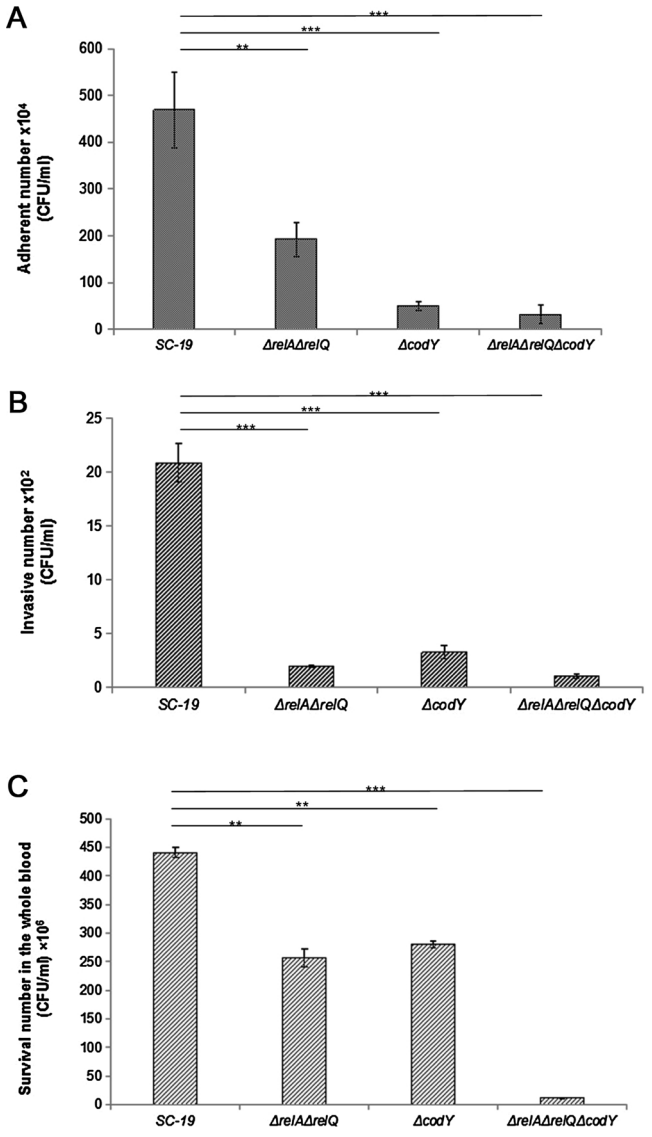


Fig. 4. Identification of the adherent, invasive and resistance to the whole blood killing ability of SC-19, Δ*relA*Δ*relQ*, Δ*codY* and Δ*relA*Δ*relQ*Δ*codY* strains. (A) The adherence ability to HEp-2 cells among SC-19, Δ*relA*Δ*relQ*, Δ*codY* and Δ*relA*Δ*relQ*Δ*codY* strains. (B) The invasion ability to HEp-2 cells among SC-19, Δ*relA*Δ*relQ*, Δ*codY* and Δ*relA*Δ*relQ*Δ*codY* strains. (C) The survival number of SC-19, Δ*relA*Δ*relQ*, Δ*codY* and Δ*relA*Δ*relQ*Δ*codY* strains in the mouse whole blood. Data were shown in means ± SD. (**$p < 0.01$, ***$p < 0.001$).

levels of *relA* in WT *S. suis* and the Δ*codY* mutant, and found that *relA* was down-regulated in Δ*codY* (Fig. 7D). These results suggest that CodY controls the expression of *relA*, as a positive regulatory protein, by binding to the promoter of *relA* in a GTP-independent manner.

4. Discussion

The (p)ppGpp synthetases and CodY are two important global regulators of bacteria, and are reported to not only regulate various cellular functions, including the stress response, metabolism and adaptation to changing environments, but also to play important roles in the virulence of some pathogens (Sonenshein, 2005; Jain et al., 2006; Syal et al., 2015; Brinsmade, 2017). In previous studies, we identified the (p)ppGpp synthetases (RelA and RelQ) and CodY of *S. suis* (Feng et al., 2016; Zhu et al., 2016). To further investigate the interrelationship of these two important global regulators and their co-regulation on morphology and pathogenesis, we constructed a Δ*relA*Δ*relQ*Δ*codY* mutant, and evaluated its growth, morphology, and pathogenicity compared with those of the WT and single mutants. We also investigated the cross-regulation of these two regulators

We first evaluated the growth and morphology of WT and three mutants. Knockout of the (p)ppGpp synthetase genes and/or *codY* slowed bacterial growth, and the growth of the Δ*relA*Δ*relQ*Δ*codY* mutant was slowest of all the strains (Fig. 1B). Interestingly, the chain length of Δ*relA*Δ*relQ*Δ*codY* was partly restored to the WT length, but clearly differed from those of Δ*relA*Δ*relQ* and Δ*codY* (Fig. 2). The capsule of Δ*relA*Δ*relQ*Δ*codY* was thick and rough (Fig. 3). (p)ppGpp plays important roles in the bacterial life cycle by regulating the translational machinery, the genes required for growth and division, and so on (Magnusson and Farewell, 2005; Potrykus and Cashel, 2008; Boutte and rosson, 2013, whereas CodY is responsible for changes in BCAAs and regulates global gene expression, including that of the *cps* gene cluster (Sonenshein, 2005; Levdikov et al., 2006). Our results suggest that knockout of both the (p)ppGpp synthetase genes and *codY* in a single strain aggravated the growth defect seen in the individual mutants. However, the restored chain length and thick capsule of Δ*relA*-Δ*relQ*Δ*codY*, contrary to the tendencies observed in the single-gene-knockout strains, suggests that the (p)ppGpp synthetases and CodY restrict each other's effects on the expression of capsular polysaccharide (*cps*) and division-related genes. According to a qRT-PCR assay, the expression of *cps* gene cluster was dysregulated in these mutants. For example, *cps2H* was down-regulated in Δ*relA*Δ*relQ* and Δ*codY*, but up-regulated in the Δ*relA*Δ*relQ*Δ*codY* mutant. However, some genes in the *cps* gene cluster (*cps2A*) showed reduced expression in all the mutants, whereas the expression of *cps2J* was up-regulated in Δ*relA*Δ*relQ* but down-regulated in the Δ*codY* and Δ*relA*Δ*relQ*Δ*codY* mutants. This dysregulation may explain the increased thickness of the capsule and its rough and irregular shape in the Δ*relA*Δ*relQ*Δ*codY* mutant. The chain length of *S. suis* cocci is partly determined by its cell division and chromosomal separation processes (Evans et al., 2014), and the restored short chain in Δ*relA*Δ*relQ*Δ*codY* might be attributable to the over-expression of DivIVA in this mutant (Fig. 6). In total, the morphology and gene expression analysis suggested that both (p)ppGpp synthetases and CodY are essential for maintaining the growth, but these two regulators also restrict each other's effects on capsular polysaccharide synthesis and cell division.

The pathogenicity-related phenotypes of Δ*relA*Δ*relQ*Δ*codY* mutant were evaluated by assaying its adherence, invasion, and resistance to whole-blood killing *in vitro*, and its lethality and colonization in mice (Fig. 5). All these tests showed consistent results, and the pathogenicity and resistance capacities of Δ*relA*Δ*relQ*Δ*codY* were clearly lower than those of the WT and the single-gene mutants. Several main virulence factors reported in *S. suis* were tested with a qPCR analysis (Fig. 6). Among the genes encoding these virulence factors, *fbps*, *eno*, and *gapdh* are also important in adhesion (Edwards et al., 2004; Kinoshita et al., 2008; Tang et al., 2013), and *arcA* is involved in resistance and colonization (Buettner et al., 2008; Lasaro et al., 2014). The knockout of *fbps*, *mrp*, *epf*, and/or *sao* reduces the virulence of *S. suis* in the mouse, zebrafish, and pig (Vecht et al., 1996; Roy et al., 2014; Li et al., 2017a, b). As shown in Fig. 6, the expression of these virulence factors was strongly reduced in the Δ*relA*Δ*relQ*Δ*codY* mutant, consistent with its pathogenicity phenotypes. These results suggested that both (p)ppGpp synthetases and CodY co-regulated the expression of virulence factors, and were essential for maintaining virulence. In addition, knockout of these two regulators was the superimposed effect on reduced expression of virulence.

The association between CodY and the (p)ppGpp synthetases and their effects on virulence were first studied extensively in *L. monocytogenes* (Bennett et al., 2007), but knockout of these two regulators in it showed restored virulence, which was different from our results in *S. suis*. GTP is well-known as the substrate of (p)ppGpp synthetase, and in *L. monocytogenes*, GTP was shown to be a ligand of CodY, promoting its DNA-binding ability. The knockout of *relA*, which encodes a (p)ppGpp

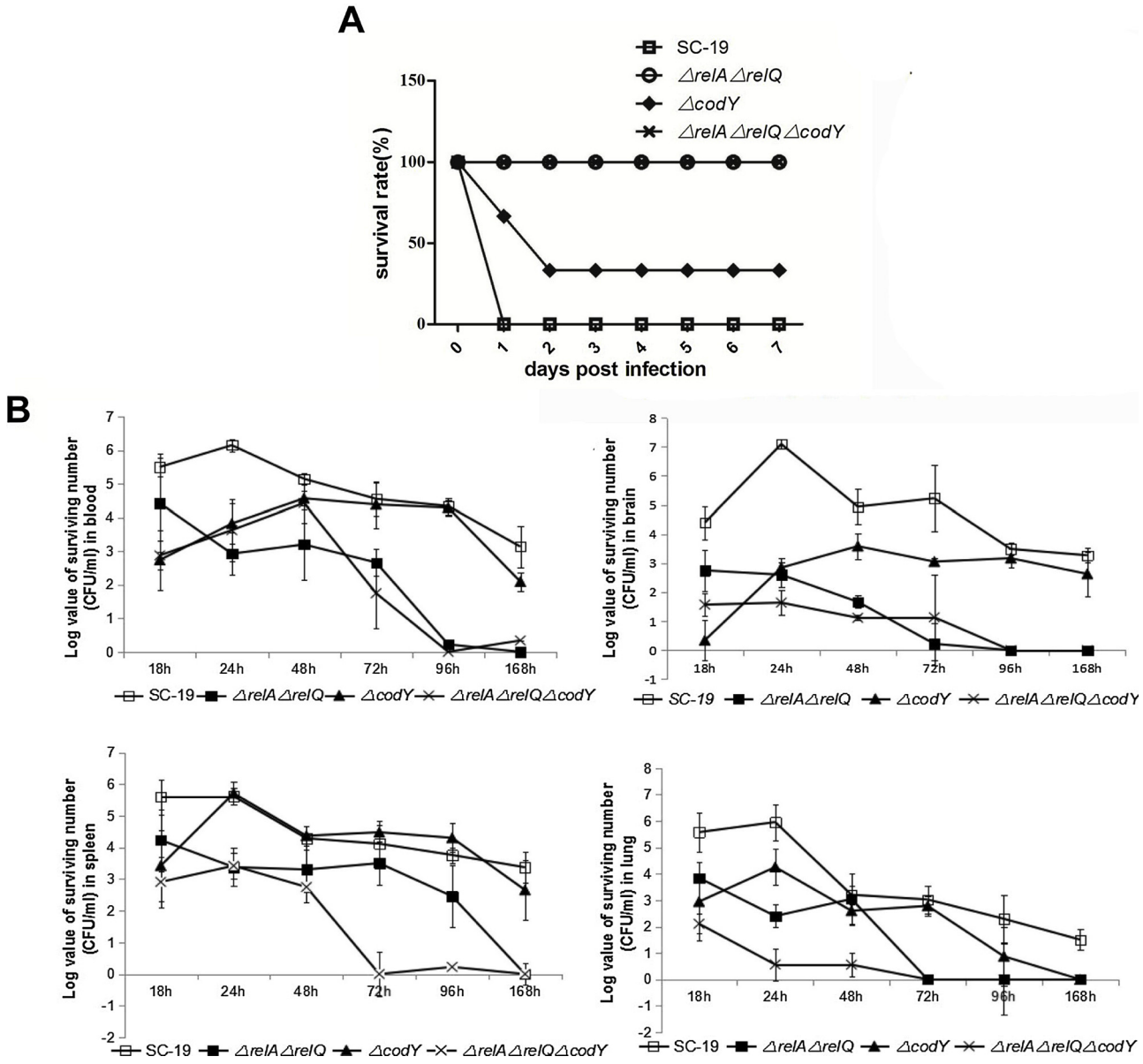


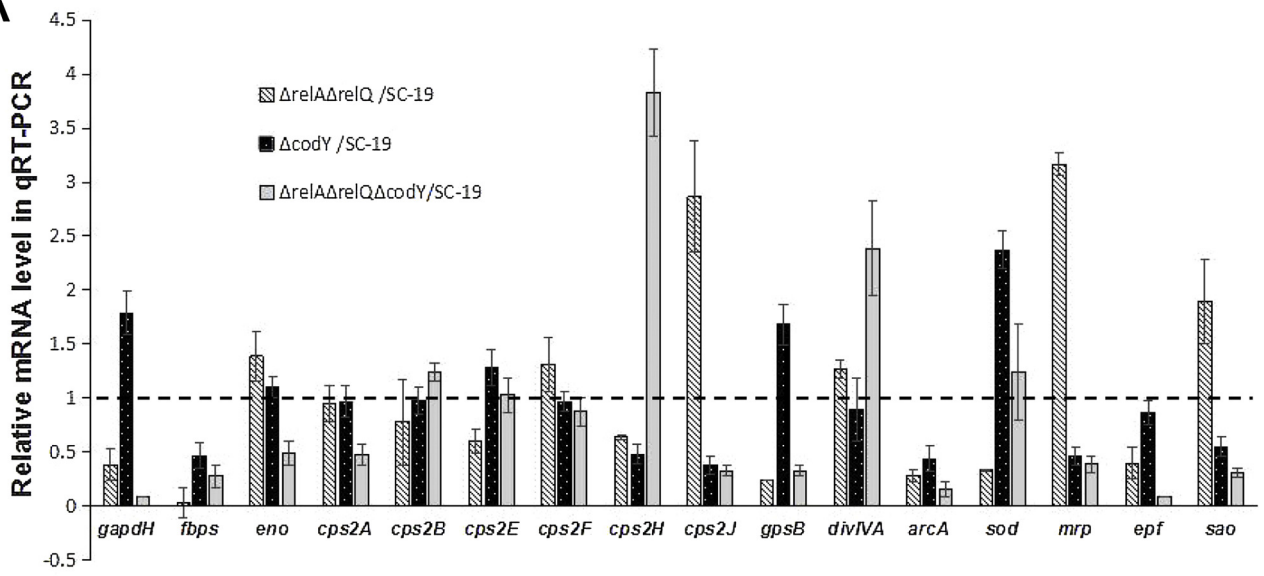
Fig. 5. Virulence detection of SC-19, Δ*relA*Δ*relQ*, Δ*codY* and Δ*relA*Δ*relQ*Δ*codY* strains. (A) Kunming female mice in each group were intraperitoneally injected with 5×10^7 CFU of SC-19 (□), Δ*relA*Δ*relQ* (○), Δ*codY* (◆) and Δ*relA*Δ*relQ*Δ*codY* (×) strains respectively. The mortality of mice was recorded in one week. (B) Choosed the challenge dose of approximately 5×10^7 CFU/mouse to inject mice, dissected them and isolated bacteria from blood, brain, spleen and lung organs at desired times of 18 h, 24 h, 48 h, 72 h, 96 h and 168 h. Data were means ± SD of bacterial colonies from six mice.

synthetase, increased the GTP pool in the bacterial cells and contributed to the repression of CodY to its regulon, causing the avirulence of the Δ*relA* mutant. The introduction of Δ*codY* into the Δ*relA* strain disinhibited the expression of the virulence-related genes controlled by CodY and restored the virulence of the Δ*relA* strain (Bennett et al., 2007). To explain the different virulence phenotypes in *S. suis* and *L. monocytogenes*, we try to find the link between CodY and the (p)ppGpp synthetases in *S. suis*. Interestingly, we confirmed that the DNA binding of CodY was independent of GTP (Fig. 7), and CodY positively and GTP-independently controls the expression of *relA* in *S. suis*, which suggest that a new molecular mechanism underlies the co-regulation of gene expression by CodY and (p)ppGpp synthetases. As our previous studies, many virulence-related genes were regulated by (p)ppGpp and/or CodY (Feng et al., 2016; Zhu et al., 2016). Hence, it is different from in *L. monocytogenes*, GTP was not the linker of CodY the (p)ppGpp synthetases, and the co-regulation of CodY and (p)ppGpp synthetases on virulence is a superimposed effect. Therefore, introducing a *codY* mutant into the Δ*relA* mutant lead to highly reduced growth and virulence factors expression, and generated a highly attenuated mutant.

As reported in *S. mutans*, reducing the GTP levels did not alleviate the repression of CodY, suggesting that *S. mutans* CodY is not activated by GTP (Lemos et al., 2008). Similarly, GTP does not act as a cofactor of CodY in *S. pneumoniae* (Hendriksen et al., 2008). A sequence analysis showed that the (p)ppGpp synthetases and CodY are highly conserved in the streptococci, so this co-regulatory mechanism may also exist in these bacteria.

In this study, we characterized the morphology and pathogenicity of the Δ*relA*Δ*relQ*Δ*codY* mutant of *S. suis*, and showed that it is highly attenuated. We found that CodY regulates the expression of *relA* GTP-independently in *S. suis*, thus clarifying a new co-regulation relationship between the (p)ppGpp-mediated stringent response and CodY on morphology and pathogenesis in bacteria.

A



B

Gene	Fold Change ratio (±SD)		
	ΔrelAΔrelQ /SC-19	*ΔcodY /SC-19*	*ΔrelAΔrelQΔcodY/SC-19*
gapdH	**0.38±0.14**	***1.79±0.20***	**0.09±0.00**
fbps	**0.03±0.14**	**0.47±0.12**	**0.28±0.10**
eno	1.39±0.23※	1.10±0.09※	**0.49±0.11**
cps2A	0.95±0.17※	0.97±0.14※	**0.47±0.10**
cps2B	0.78±0.40※	0.98±0.13※	1.24±0.08※
cps2E	0.60±0.11※	1.28±0.17※	1.03±0.16※
cps2F	1.31±0.25※	0.97±0.09※	0.87±0.13※
cps2H	0.64±0.02※	**0.48±0.09**	***3.83±0.40***
cps2J	***2.87±0.52***	**0.37±0.08**	**0.33±0.05**
gpsB	**0.24±0.01**	***1.68±0.19***	**0.33±0.05**
divIVA	1.26±0.08※	0.89±0.29	***2.39±0.44***
arcA	**0.28±0.06**	**0.44±0.12**	**0.15±0.07**
sod	**0.33±0.01**	***2.37±0.17***	1.24±0.44※
mrp	***3.17±0.11***	**0.46±0.08**	**0.39±0.07**
epf	**0.40±0.15**	0.87±0.11※	**0.08±0.00**
sao	***1.90±0.39***	0.55±0.09※	**0.31±0.04**

Bolded text indicated the gene was downregulated when compred to that of SC-19.
Italics text indicated the gene was upregulated when compared to that of SC-19.
※ Fold change was not significant in qRT-PCR confirmation.

Fig. 6. Fold change ratios of *ΔrelAΔrelQ*, *ΔcodY* and *ΔrelAΔrelQΔcodY* mutants/SC-19 by qRT-PCR. (A) The mRNA level of *gapdH*, *fbps*, *eno*, *cps2A*, *cps2B*, *cps2E*, *cps2F*, *cps2H*, *cps2J*, *gpsB*, *divIVA*, *arcA*, *sod*, *mrp*, *epf* and *sao* of mutants were shown as relative expression ratios compared to that of SC-19. (B) Data of differentially expressed genes were shown as relative expression ratios compared to that of SC-19. Data from three independent assays are presented as the means ± SD.

Acknowledgments

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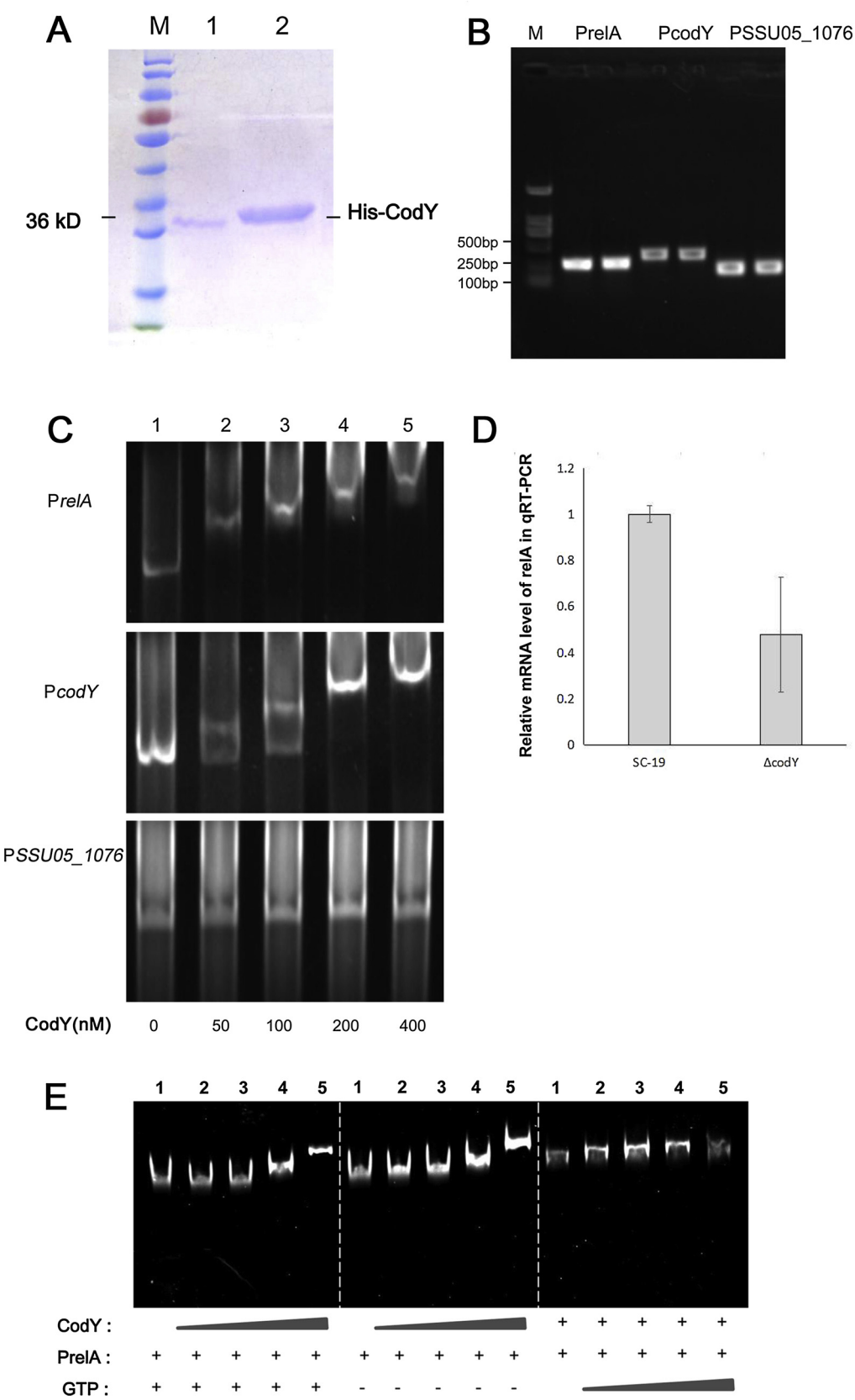

Fig. 7. The EMSAs results of CodY proterin interacting with promoters and GTP. (A) Expression and purification of His-CodY protein, which was 36 kD. (B) Identification of promoter fragments of P*relA*, P*codY* and P*SSU05_1076* by PCR. (C) CodY could interact with P*relA* and the positive sample P*codY* but not the negative sample P*SSU05_1076*. (D) Relative mRNA level of *relA* was down-regulated in qRT-PCR. (E) The affinity of GTP and CodY. Increasing the concentration of CodY protein with/without the same level of GTP showed there was not significant change in migration rate. The same concentration of CodY protein with increasing GTP level also did not display the faster speed of mobility.

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