Function of Global Regulator CodY in *Bacillus thuringiensis* BMB171 by Comparative Proteomic Analysis

Mingxia Qi¹, Fei Mei¹, Hui Wang¹, Ming Sun¹, Gejiao Wang¹, Ziniu Yu¹, Yeonho Je², and Mingshun Li*¹

¹State Key Laboratory of Agricultural Microbiology, Huazhong Agricultural University, Hongshan District, Wuhan 430070, P.R. China
²School of Agricultural Biotechnology, College of Agriculture and Life Sciences, Seoul National University, Seoul 151-742, Republic of Korea

CodY is a highly conserved regulatory protein in low G+C gram-positive bacteria that regulates genes involved in sporulation and stationary-phase adaptation. *Bacillus thuringiensis* is a gram-positive bacterium that forms spores and parasporal crystals during the stationary phase. To our knowledge, the regulatory mechanism of CodY in *B. thuringiensis* is unknown. To study the function of CodY protein in *B. thuringiensis*, BMB171codY was constructed in a BMB171 strain. A shuttle vector containing the ORF of cry1Ac10 was transformed into BMB171 and BMB171codY, named BMB171cry1Ac and BMB171codYcry1Ac, respectively. Some morphological and physiological changes of codY mutant BMB171codYcry1Ac were observed. A comparative proteomic analysis was conducted for both BMB171codYcry1Ac and BMB171cry1Ac through two-dimensional gel electrophoresis and MALDI-TOF-MS/MS analysis. The results showed that the proteins regulated by CodY are involved in microbial metabolism, including branched-chain amino acid metabolism, carbohydrate metabolism, fatty acid metabolism, and energy metabolism. Furthermore, we found CodY to be involved in sporulation, biosynthesis of poly-β-hydroxybutyrate, growth, genetic competence, and translation. According to the analysis of differentially expressed proteins, and physiological characterization of the codY mutant, we performed bacterial one-hybrid and electrophoretic mobility shift assay experiments and confirmed the direct regulation of genes by CodY, specifically those involved in metabolism of branched-chain amino acids, ribosomal recycling factor FRR, and the late competence protein ComER. Our data establish the foundation for in-depth study of the regulation of CodY in *B. thuringiensis*, and also offer a potential biocatalyst for functions of CodY in other bacteria.

Keywords: *Bacillus thuringiensis*, branched-chain amino acids, CodY, FRR, poly-β-hydroxybutyrate, proteomics

**Introduction**

CodY is a highly conserved regulatory protein in low G+C gram-positive bacteria that regulates many virulence genes in pathogenic microorganisms [12, 14, 26]. IDAP-Seq methods (genome-wide identification of protein-binding regions in vitro) have been used successfully to identify CodY-regulated genes in *Bacillus subtilis*, *Staphylococcus aureus*, *Clostridium difficile*, and *Bacillus anthracis* [2, 7, 14, 29]. It has been demonstrated that CodY regulates genes involved in the degradation of macromolecules, transport of nutrients, microbial metabolism, antibiotic synthesis, and chemotaxis [3, 23, 30]. CodY is a dimeric protein that contains a winged helix-turn-helix DNA-binding motif and ligand-binding GAF domain [26]. The effectors of CodY are GTP and the branched-chain amino acids (BCAAs) isoleucine, leucine, and valine. GTP and BCAAs increase synergistically the affinity of CodY for its DNA target sites [20, 42].

Current research on CodY focuses mainly on binding sites, effectors, and the regulation of many virulence genes in *B. subtilis* and some pathogenic microorganisms, including *S. aureus*, *C. difficile*, and *B. anthracis* [2, 7, 12, 14, 26, 29].
contrast, the regulatory mechanisms of CodY in *B. thuringiensis*, the primary bacterial entomopathogen that produces proteinaceous parasporal crystalline toxin inclusions used in biocontrol worldwide, is unknown. To study the function of CodY protein in *B. thuringiensis*, a *codY* knockout *B. thuringiensis* strain, BMB171*codY*, was constructed in the BMB171 strain. A shuttle vector containing the ORF of *cry1Ac* and *B. thuringiensis* codY protein in biocontrol worldwide, is unknown. To study the function of the ORF of insecticidal crystal protein gene *cry1Ac*10 was used to transform BMB171 and BMB171*codY*, named BMB171*cry1Ac* and BMB171*codY cry1Ac*, respectively.

Flasks containing 50 ml of culture medium were incubated at 28°C. Cell samples were taken and measured at every 2 h. We determined the cell growth curve at each time point by measuring the optical density at 600 nm using a Shimadzu UV-1201 spectrophotometer (Osaka, Japan). The growth curve was prepared with the mean optical densities of triplicate bacterial cultures at each time point. The plate counting dilution method was also used to determine the growth curves of BMB171cry1Ac and BMB171*codY cry1Ac*. The appropriate dilution degree of bacteria liquid was made in order to guarantee the plate had 30–300 bacterial colonies.

Transmission Electron Microscopic Observation

The bacterial pellets from cultures of BMB171*cry1Ac* and BMB171*codY cry1Ac* after 28 h were harvested by centrifugation at 10,000 ×*g* at 4°C for 5 min. In brief, the pellets were washed with phosphate-buffered saline (PBS), and suspended in 0.1 M Sorensen buffer containing 2.5% (v/v) glutaraldehyde. After fixation, dehydration, embedding, and ultrathin sectioning were performed. Sections were stained in 1% (w/v) uranyl acetate and lead citrate solution. The stained samples were examined using FEI Tecnai G 20 TWIN (Portland, OR, USA) operating at 80 kV.

**Materials and Methods**

**Bacterial Strain and Culture Conditions**

In this study, we used bacterial strains *B. thuringiensis* BMB171, a plasmid-cured, acrystalliferous strain [27]; *Escherichia coli* DH5α; and *E. coli* BL21. Each was grown in Luria–Bertani (LB) broth or on LB agar plates. *E. coli* strains DH5α and BL21 were grown at 37°C. The *B. thuringiensis* strains were grown at 28°C. Peptone medium (per liter culture medium contains peptone 10 g, glucose 5 g, yeast extract 1 g, MgSO4·7H2O 0.3 g, FeSO4·7H2O 0.02 g, ZnSO4·7H2O 0.02 g, and MnSO4·7H2O 0.02 g) was used to quickly induce spore formation in *B. thuringiensis*.

**Construction of codY Knockout Mutant BMB171*codY cry1Ac***

Using Taq DNA polymerase (Takara Biotechnology, China), an upstream region and a downstream region of the *codY* gene were amplified from BMB171 genomic DNA with the primer pairs P1 (5′AGTGAATTCACAGGGAGATTTCCGATTC)/P2 (5′ATTGGA TTCACCTAGACGCTCACCGCCAC) and P3 (5′CGCGGATCC TATTAACGTCGTTTGC)/P4 (5′GCTCTAGACGATTTCCGATGCCTT). The amplified fragments were 1.0 and 1.1 kb in length. To construct the knockout vector pBMB0413, both fragments and the spectinomycin-resistant gene (1.6 kb) were inserted into the multiple cloning sites of temperature-sensitive carrier pH7304-TS [1]. BMB171 was transformed with pBMB0413. The *codY knockout mutant was screened for erythromycin and spectinomycin resistance under incubation at 42°C. A resulting clone was chosen and named as BMB171*codY*. Shuttle vector pBMB43-304 containing the ORF of insecticidal crystal protein gene *cry1Ac*10 was used to transform BMB171 and BMB171*codY*, named BMB171*cry1Ac* and BMB171*codY cry1Ac*, respectively.

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**Determination of PHB Concentration and SDS-PAGE Analysis**

We determined the bacterial poly-β-hydroxybutyrate (PHB) content by the spectrophotometric method described by Law and Slepecky [25].

For protein extraction, we collected cells representing different growth phases, specifically 7, 10, and 22 h, and 12, 20, and 30 h for strains BMB171*cry1Ac* and BMB171*codY cry1Ac*, respectively. Bacterial cultures were boiled for 10 min. The cell debris was removed by centrifugation at 10,000 ×*g* for 5 min. The samples were subjected to 12% SDS-PAGE and stained using Coomassie blue [17].

**2-D Protein Electrophoresis and Image Generation**

We collected protein samples representing the mid-log phase, early-stationary phase, and late-stationary phase at 7, 10, and 19 h, and 12, 20, and 24 h for strains BMB171*cry1Ac* and BMB171*codY cry1Ac*, respectively. Protein extractions were conducted as described by Gong et al. [18]. The bacterial cells were harvested by centrifugation at 10,000 ×*g* at 4°C for 5 min. The cells were then resuspended and washed three times with ice-cold PBS. The cells were then suspended in lysis buffer with a protease inhibitor cocktail (Sigma, Missouri, USA) and 1% (w/v) DTT, and then treated by ultrasonication for 10 min. DNase I and RNase A were added to the lysate at a final concentration of 1 mg/ml and 0.25 mg/ml, respectively. The mixture was incubated at 28°C for 1 h. The cell debris was removed by centrifugation at 12,000 ×*g* at 4°C for 20 min. Excess salts were removed from the crude protein samples using the ReadyPrep 2-D cleanup Kit (Bio-Rad Laboratories,
Pennsylvania, USA). Protein concentrations were determined by the Bradford method [5]. Purified protein samples were run on isoelectric focusing (IEF) IEF-strips (pH 4–7, 17 cm; Bio-Rad). Samples were loaded by active rehydration under an electric field of 50 V for 2 h. First, the IEF was run at 200 V for 1 h. Second, the voltage was increased linearly and maintained at 1,000 V for 1 h. Third, the voltage was raised rapidly and held at 10,000 V for 5 h. Last, the voltage was increased linearly to a maximum of 90,000 V [8, 45]. After IEF, the focused strips were placed first in an equilibration solution containing 1% (w/v) dithiothreitol for 15 min, and then in the equilibration solution with 4% (w/v) iodoacetamide for 15 min. The equilibrated strips were placed on 12% SDS-PAGE gels and covered with 0.5% low melting point agarose containing trace amounts of Coomassie bromophenol blue G250. The gels were run in a Bio-Rad Dodeca Cell system at a constant voltage of 80 V for 15 min, and then at 200 V until the marker dye reached the bottom of the gels. After electrophoresis, the gels were stained with silver nitrate [4], scanned with the GS-800 calibrated densitometer (Bio-Rad), and analyzed with the PD Quest software (ver. 8.0; Bio-Rad). Protein profiles with good convergent validity of protein spots, clear background, uniform distribution, and good reproducibility were used for proteomics analysis. Protein samples at each stage were carried out three times in repeated experiments. At least three gels were analyzed for each phase. Proteins were considered differentially expressed when the average values exceeded the thresholds of 2-fold differences and a p-value < 0.05.

**Protein Identification by MALDI-TOF MS/MS**

The differentially expressed protein spots were excised manually from the gels and placed in Eppendorf tubes. Briefly, gel pieces were destained with a solution of 30 mM potassium ferricyanide and 100 mM sodium thiocyanate (1:1 (v/v)) for 20 min at room temperature. The spots were incubated in 0.2 M NH₄HCO₃ for 20 min and then lyophilized. Each spot was digested overnight in 5 µl (2.5–10 ng/µl) of trypsin (Promega) at 37°C. The peptides were extracted three times with 60% acetonitrile/0.1% trifluoroacetic acid. The extracts were pooled and dried completely in a vacuum centrifuge.

MALDI-TOF MS and MS/MS analyses were performed at Shanghai Applied Protein Technology Co. Ltd. MS and MS/MS data for protein identification were obtained by using the 4800 Plus MALDI TOF/TOF Analyzer (Applied Biosystems). Combined peptide mass fingerprinting and MS/MS queries were performed by using the MASCOT search engine 2.2 (Matrix Science, Ltd.) Search parameters were set as follows: tolerance to 100 ppm peptide mass variance, trypsin cleavage to one missed cleavage allowed, carbamidomethylation set as fixed modification, oxidation of methionine allowed as variable modification, and MS/MS fragment tolerance set to 0.4 Da. A MASCOT confidence interval of 95% was used for further manual validation.

**Real-Time PCR Analysis of Differentially Expressed Proteins**

The total RNA was isolated with Trizol reagent according to the manufacturer’s instructions (Invitrogen, Grand Island, NY, USA). The RNA samples were treated with RNase-free DNase I (Invitrogen, USA). The quality and quantity were assessed in the UV spectrophotometer DU800 (Beckman Coulter, North Rhine-Westphalia, USA). Reverse transcription and real-time PCR were performed with the SYBR PrimeScript RT-PCR Kit II (Takara BioRO Inc., Shiga, Japan) following the manufacturer’s instructions.

Primers for real-time PCR were designed with the Primer V5 software (PREMIER Biosoft International, CA, USA) according to the GenBank sequences. We chose the 16S rRNA as the reference gene to normalize expression levels among different samples. All gene-specific primers are listed in Table S2. Quantitative RT-PCR was performed using an ABI Viia 7 Real-Time PCR System (PE Applied Biosystems, Foster City, CA, USA) using standard procedures (Stage 1: Initial denaturation, 95°C for 10 min; Stage 2: Amplification, 95°C for 15 sec, 58°C for 30 sec, 72°C for 40 sec, 40 cycles; Melt Curve Stage: 95°C for 15 sec, 60°C for 1 min). Negative (no template) controls were included in each PCR run. Each experiment was tested in triplicate. Gene expressions were normalized by 2^−ΔΔCt analysis [28].

**Bacterial One-Hybrid System and EMSA**

Library vector pTRG-codY and reporter vector PBXcmT-gene were constructed and co-transformed into the XL-blue strain [19]. The reporter strains were selected on agar plates containing 15 mM 3-AT (3-amino-1, 2, 4-triazole) and 16 µg/ml streptomycin. Agar plates that did not contain 3-AT and streptomycin were used as controls. Co-transformants containing PBXcmT-Rv2031p and pTRG-Rv3133c were used as positive controls [32]. The purified protein CodY (750 µg/ml), 100-200 bp fragments (100 ng/µl) of target genes, binding buffer (5x), poly L-lysine (0.1 µg/µl), and GTP and BCAAs with the final concentration of 2 mM and 10 mM, respectively, were mixed and incubated at 28°C. After 30 min, the binding reaction mixtures were loaded on a 6.5% native polyacrylamide gel prepared in Tris-glycine buffer.

**Results and Discussion**

We constructed a codY deletion clone from B. thuringiensis strain BMB171 by a gene displacement method. Some physiological changes were observed. This study was conducted to establish a comparative proteomic analysis between different growth phases of BMB171cry1Ac and BMB171codY cry1Ac. The physiological characterization of the codY mutant, metabolic changes of BCAAs and PHB, translation-related proteins ribosomal recycling factor FRR related to protein synthesis, and late competence protein ComER are discussed below.

**Morphological and Physiological Changes of codY Mutant Bacillus thuringiensis Strain**

Strain BMB171cry1Ac formed spores and parasporal
crystals during the stationary phase, whereas BMB171codYcry1Ac formed neither spores nor parasporal crystals (Fig. 1A). SDS-PAGE revealed a distinct difference at ~130 kDa (Cry1Ac), where the protein toxin was present for BMB171cry1Ac but not BMB171codY at entry into the stationary phase (Fig. 1B). The growth of BMB171cry1Ac differed from that of BMB171codYcry1Ac (Fig. 1C). BMB171cry1Ac and BMB171codYcry1Ac entered the stationary phase at 10 and 20 h, respectively. The maximum OD_{600} values of the strains were 10.00 and 14.00. Considering the effect of different morphological characteristics on the refractive index, we used a plate counting dilution method to determine the growth differences between BMB171cry1Ac and BMB171codYcry1Ac (Fig. 1D). The growth trend using the OD_{600} values and plate counting were similar. PHB accumulation in cells played an important role in cell metabolism [8]. In BMB171cry1Ac, the PHB yield accumulated at the mid-log phase, gradually decreased, and reached its minimum at the late-stationary phase. In contrast, the PHB yield accumulated and remained stable at the stationary phase in BMB171codYcry1Ac (Fig. 1D). In short, morphological and physiological changes of the codY mutant were observed. CodY influenced genes associated with growth and sporulation. Additionally, PHB accumulation in cells was regulated by CodY in B. thuringiensis.

2-DE Analysis, Identification, and Functional Classification of Differentially Accumulating Proteins

We extracted total proteins from BMB171cry1Ac and BMB171codYcry1Ac at three different stages (mid-log phase, early-stationary phase, and late-stationary phase) and determined protein profiles for each by 2-DE (Fig. 2).

**Fig. 1.** Characterization of BMB171cry1Ac and BMB171codYcry1Ac. (A-1) Transmission electron microscopic observations of BMB171cry1Ac. Bacterial cells were sampled at 30 h. Spore and ICPs are indicated by arrows. (A-2) Transmission electron microscopic observations of BMB171codYcry1Ac. Bacterial cells were sampled at 30 h. A bright oval spot indicated by an arrow refers to a cross-section of the PHB granules. Scale bars in A-1 and A-2 equal 0.5 µm. (B) SDS-PAGE analysis of BMB171cry1Ac and BMB171codYcry1Ac proteins. M: Unstained protein molecular weight marker; Lane 1: proteins of BMB171cry1Ac-7 h; Lane 2: proteins of BMB171codYcry1Ac-12 h; Lane 3: proteins of BMB171cry1Ac-10 h; Lane 4: proteins of BMB171codYcry1Ac-20 h; Lane 5: proteins of BMB171cry1Ac-22 h; Lane 6: proteins of BMB171codYcry1Ac-30 h. (C) BMB171cry1Ac and BMB171codYcry1Ac growth curves. A bar for each sampling time point denotes standard error of the mean from three batches. (D) PHB concentration of BMB171cry1Ac and BMB171codYcry1Ac at different times. Sampling times and error bars are shown in the figure.
Fig. 2. Protein profiles of BMB171cry1Ac and BMB171codYcry1Ac on 2-DE gels.
The identified protein spots are labeled and indicated by arrowheads. Protein abbreviations in red color represent up-regulated expression, down-regulated expression is in green, and no change in expression is in black. (A-1) and (A-2) The protein profiles of BMB171cry1Ac-7 h and BMB171codYcry1Ac-12 h, respectively, sampled in mid-log phase. (B-1) and (B-2) The protein profiles of BMB171cry1Ac-10 h and BMB171codYcry1Ac-20 h, respectively, in the early-stationary phase. (C-1) and (C-2) The protein profiles of BMB171cry1Ac-19 h and BMB171codYcry1Ac-24 h, respectively, in the late-stationary phase. The isoelectric focusing was run in a pH range of 4-7. The protein molecular weight standard is given to the left of the gel images.
Most proteins were within pH 4–7; and the molecular masses were in the range of 10–100 kDa. Forty-three proteins were significantly different in intensity (2-fold or more). Each was identified by MALDI-TOF-MS/MS, revealing 36 matched known proteins (Table S1). The identified proteins were grouped into four classes: metabolism, genetic information processing, environmental information processing, and cellular processes, based on Kyoto Encyclopedia of Genes and Genomes Orthology (KO) protein function annotations. The majority of the identified proteins (75%) were involved in the metabolism category, of which 28% were those involved in carbohydrate metabolism, 21% were involved in amino acid metabolism, and 14% were involved in energy metabolism (Fig. S2). The analysis of a genome-wide identification of CodY binding regions in *B. subtilis*, *S. aureus*, *C. difficile*, and *B. anthracis* revealed the majority genes were involved in metabolism [2, 7, 14, 29]. These data indicate that CodY regulates proteins involved in metabolic activity.

Proteomic Analyses of Abundance Changes of Some Proteins

At the three different phases tested between BMB171cry1Ac and BMB171codYcry1Ac, heat maps of differentially expressed proteins showed expression changes of some proteins involved in the metabolism of BCAAs and PHB, sporulation, growth, genetic competence, and translation (Fig. 3). Real-time quantitative PCR was carried out to verify the differences in transcription. The results were consistent with 2-DE data (Table S3).

Proteins Involved in Growth

The *codY* deletion mutant strain, BMB171codYcry1Ac, could not form spores or parasporal crystals. Additionally, the log phase was extended and the stationary period was delayed (Fig. 1). According to MALDI-TOF-MS analyses and protein expression changes, we found that proteins DivIVA and MreB increased in expression from the early-stationary phase to the late-stationary phase (Fig. 3). DivIVA, a coiled-coil, tropomyosin-like protein, is an important protein in gram-positive bacteria cell division, as it localizes to division sites and is retained at the cell poles after division in *B. subtilis* [41]. In *B. subtilis*, divIVA mutant strains are inhibited in cell division initiation [16]. The main cell wall component, peptidoglycan, and the actin-like MreB cytoskeleton are major determinants of cell shape in rod-shaped bacteria [15]. MerB has a high similarity to eukaryotic actin filaments in amino acid sequence, assembly, structure, and function. Furthermore, MreB has a dual role in chromosome segregation and in the formation of rod cell shape in *B. subtilis* [11]. Increases in the expression of DivIVA and MreB may lead to the delayed log phase and the increase of biomass.

Proteins Involved in PHB Metabolism

PHB provides energy and a carbon source for bacterial reproduction or stress tolerance [40]. In our study, the PHB yield in BMB171codYcry1Ac continuously accumulated and remained in the cell at the stationary phase, whereas in BMB171cry1Ac, the PHB yield accumulated and remained stable at the stationary phase (Fig. 1D). Previous studies revealed that the energy for synthesis of parasporal crystals originates from PHB hydrolysis in *B. thuringiensis* [18]. Two proteins, 3-hydroxybutyryl-CoA dehydratase Crt and 4-aminobutyrate aminotransferase GabT, important enzymes in PHB biosynthesis, were up-regulated at all three stages.
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in the codY mutant strain (Fig. 3). Crt participates in butyrate metabolism and can catalyze acetoacetyl-CoA to 3-hydroxybutyryl-CoA. Synthesis of PHB from 3-hydroxybutyryl-CoA is catalyzed by the poly(R)-hydroxyalkanoic acid synthase subunit PhaC. GabT, which participates in gamma-aminobutyric acid metabolism, catalyzes aminoacetate to succinate semialdehyde, which may promote the synthesis of crotonyl CoA as the precursor of PHB (Fig. 4). PHB begins to accumulate during the exponential phase, and is utilized in the stationary phase in *B. thuringiensis* as a carbon and energy storage material [9]. Proteins involved in the PHB degradation pathway have not yet been identified in our study. A PHB granule is surrounded by a layer of phospholipids and proteins, with phasins (PhaP) as the predominant compound [13]. Overexpression of PhaP results in the formation of many small granules in cells [33]. In contrast, a deletion of the phaP gene results in the formation of only one or two large granules [44]. Decreased expression of PhaP in the mutant strain led to a small number, but larger PHB granules.

Proteins Involved in Sporulation

Previous studies revealed that CodY regulates several genes (spo0A, rapA, rapE, citB) involved in sporulation in *B. subtilis* [30, 35]. Spo0A, which is activated by phosphorylation, is an important response regulator protein in the initiation of sporulation of bacteria [6, 24]. CitB (aconitase), a factor required for efficient late-sporulation gene expression in *B. subtilis*, may regulate GerE, which is responsible for the efficient timing of spore coat assembly [34]. The spore coat-associated protein, CotN, was decreased in all of the three phases, providing evidence for the sporulation deficiency observed in our mutants (Fig. 3). Deletion of the phaC gene from the BMB171 strain results in a sporulation-deficient phenotype [8]. The continuous accumulation of PHB in BMB171codYcry1Ac may result from uncontrolled gene expression or suppression of PHB degradation pathways.

Proteins Related to BCAAs Metabolism

BCAAs are effectors of CodY. We identified by MALDI-TOF-MS 13 proteins associated with BCAAs metabolism. IlvB, IlvC, IlvE, and LeuB, which are important enzymes in the biosynthesis of BCAAs, were up-regulated at all of the three stages in the codY mutant strain (Fig. 3). Acyl-CoA dehydrogenase (ACD), dihydrolipoamide dehydrogenase (PdhD), and hydroxymethylglutaryl-CoA lyase (HmgL) participate in the degradation of BCAAs (KEGG Orthology protein function annotations). ACD, PdhD, and HmgL...
were down-regulated in each of the three phases (Fig. 3). These results suggest that CodY can regulate BCAAs metabolism. Enzymes involved in the biosynthesis of BCAAs were up-regulated, whereas those involved in BCAAs degradation were down-regulated (Fig. 5). *B. subtilis* has four transcription units devoted to BCAAs biosynthesis. All but ilvA are repressed by CodY [37–39]. Bacterial one-hybrid experiments and EMSA confirmed CodY directly regulates the promoters of the *ilv* operon in *B. thuringiensis* (Fig. 6). These data are consistent with the results in *B. subtilis* [37–39].

**Ribosomal Recycling Factor FRR**

Protein synthesis occurs in three steps: initiation, elongation, and termination. A fourth step involves the disassembly of the post-termination complex [22, 46]. Ribosomal recycling factor FRR and elongation factor EF-G split the ribosome into subunits by a mechanism requiring GTP and GTP hydrolysis [22, 47]. However, the other activities of FRR and EF-G during ribosome recycling are still controversial [21]. FRR is a multifunctional protein in prokaryotes and plays an indispensable role in synthesis of proteins [47]. In the absence of FRR, ribosomes can recognize the mRNA termination codon, but termination does not occur; another translation initiation occurs from downstream initiation codons. In the overexpressing CodY strain YBT-881-L1, FRR is down-regulated in all of the three phases (unpublished data). FRR was up-regulated in all of the three stages in *codY* mutant strain BMB171*codYcry1Ac*. CodY may directly regulate the promoter region of *frr* (Fig. 6). The relationship between CodY regulation and FRR has not been reported for other gram-positive bacteria.

**Late Competence Protein ComER**

Late competence protein ComER is reported to be associated with genetic competence in bacteria, and *comER* belongs to a member of late “*com*” genes. *comE* has been studied extensively in *Streptococcus pneumonia* [10, 43]. ComDE proteins are members of the two-component system of sensor regulators, which are essential for the signaling cascade that could lead to the development of processes responsible for genetic transformation [10]. CodY may directly regulate *comk*, another member of late “*com*” genes, in *B. subtilis* [36]. *comK*, which encodes a master regulator of genetic competence, directs late competence gene transcription [31]. In BMB171, the *comE* operon includes four genes, *comEC*, *comEA*, *comEB*, and *comER*. *comER* is arranged in the reverse orientation on the bacterial chromosome. Our bacterial one-hybrid and EMSA experiments verified a conserved 10 bp CodY binding site, TTTCAGAAAA, within *comEA* (Fig. 6). These data suggest that CodY may directly regulate “*com*” genes in *B. thuringiensis*. Unexpectedly, the expression of ComER remained unchanged in both the log and early-stationary phases and was down-regulated in the late-stationary phase (Fig. 3).

In conclusion, we conducted a functional study of the global regulator CodY in *B. thuringiensis*. CodY regulates...
many genes involved in microbial metabolism, such as amino acid metabolism, carbohydrate metabolism, fatty acid metabolism, and energy metabolism. Furthermore, CodY regulates genes involved in growth, late competence protein ComER, and BCAAs metabolism in \textit{B. thuringiensis}. In addition, CodY appears to regulate ribosomal recycling factor FRR, which has not been reported for other gram-positive bacteria. Finally, CodY appears to regulate and influence the formation of spores and parasporal crystals in \textit{B. thuringiensis}.

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