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# CodY, ComA, DegU and Spo0A Controlling Lipopeptides Biosynthesis in Bacillus amyloliquefaciens fmbJ

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**Abstract**

Bacillus amyloliquefaciens, as a biocontrol bacterium, mainly produces secondary metabolites to resist pathogenic microorganisms. In the study, B. amyloliquefaciens fmbJ could produce several antimicrobial lipopeptides (bacillomycin D, surfactin, and fengycin). In order to clarify the influence of transcriptional regulatory genes (codY, comA, degU, and spo0A) regulating the biosynthesis of lipopeptides, especially bacillomycin D, these genes in fmbJ were knocked out. The results showed that the productions of bacillomycin D were significantly reduced compared with that of fmbJ. The changes of lipopetides production in fmbJ with the genes deleted were then analyzed by comparative transcriptomics. Their deletion induced great changes in the levels of transcripts specifying metabolic pathways, quorum sensing system and substance transport system in fmbJ. Moreover, overexpression of these genes improved the productions of bacillomycin D. In particular, the overexpression of spo0A enhanced bacillomycin D yield up to 648.9 ± 60.9 mg/L from 277.3 ± 30.5 mg/L. On the contrary, the yields of surfactin in fmbJΔcodY and fmbJΔdegU were significantly improved. And, the regulatory factor CodY had no significant effect on the synthesis of fengycin. In addition, it was found that CodY had a concentration dependence on bacillomycin D synthesis. This study indicated the direction of genetic manipulation to improve the yields of antimicrobial lipopeptides and laid a theoretical foundation for the industrial production of lipopeptides.

## Introduction

*Bacillus amyloliquefaciens* (*B. amyloliquefaciens* ), a gram-positive bacterium, is widely applied as a biocontrol agent, because it can inhibit a variety of plant pathogenic microorganisms. This effect is mainly attributed to its production of a variety of secondary metabolites with antimicrobial activities (Ongena & Jacques, 2008). Previously, we reported the whole genome sequencing of *B. amyloliquefaciens* fmbJ, which is a producer of three cyclic lipopeptides (bacillomycin D, surfactin, and fengycin) (Sun, Qian, et al., 2018). These cyclic lipopeptide structures are composed of fatty acids and amino acids catalyzed by non-ribosomal peptide synthases (Ongena & Jacques, 2008). They have strong antimicrobial, antitumor, and antiviral activities, which bring them a wide range of applications in various fields, including food processing, biopesticides, pharmaceuticals, and cosmetics (Banat et al., 2010; Marr, Gooderham, & Hancock, 2006; Ongena & Jacques, 2008; Rodrigues, Banat, Teixeira, & Oliveira, 2006). Bacillomycin D has been shown to have major antifungal activity (Koumoutsi et al., 2004). However, the high production cost and low yield of bacillomycin D limited its large-scale industrial production and application. Previous studies mainly focused on the structure, biological activity and fermentation optimization of bacillomycin D (Qian et al., 2017; Sun, Li, et al., 2018; Tanaka, Ishihara, & Nakajima, 2014; Zhou et al., 2018), and there were few studies on its

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biosynthesis and regulation mechanism, which further limited the development of industrial production of bacillomycin D.

The global transcriptional regulator CodY was commonly found in gram-positive bacteria with low G-C content (Ratnayake-Lecamwasam, Serror, Wong, & Sonenshein, 2001). When dealing with environmental changes, it regulated the expression level of target genes by changing the transcription rate and could regulate the expression of 10% genes in cells, forming a huge gene regulation network (Molle et al., 2003; Sonenshein, 2005). The DNA binding activity of CodY requires two cofactors of GTP and branched-chain amino acids (BCAAs) (Belitsky, 2015; Brinsmade et al., 2014). In addition, CodY inhibited BCAAs synthesis under amino acid rich growth conditions in *B. subtilis* (Fujita, Satomura, Tojo, & Hirooka, 2014). Amino acids are the precursors of the synthesis of bacillomycin D. Does CodY regulate the synthesis of bacillomycin D by controlling the synthesis of amino acids?Studies have shown that CodY played a negative role in surfactin synthesis through directly binding to the *srfA* promoter to inhibit the expression of *srfA* (Dhali et al., 2017; Serror & Sonenshein, 1996). However, the synthetic regulation mechanism of bacillomycin D by global regulator CodY is still unclear.

In our previous study, we investigated the effects of different carbohydrates and amino acids on the productions of bacillomycin D (Qian et al., 2015; Qian et al., 2017). The addition of inulin and L-glutamine could significantly increase the yield of bacillomycin D by up-regulating the expression of *comA* , *degU* , and*spo0A* genes, which might be because these factors could promote the expression of *bmy* operon and improve the production of bacillomycin D. Moreover, studies have shown that the global regulators ComA, DegU, DegQ and the sigma factors sigmaH and sigmaB positively regulated the transcriptional activation of the *bmy* promoter in*B. amyloliquefaciens* FZB42 (Chen et al., 2009; Koumoutsi, Chen, Vater, & Borriss, 2007). But what is the network of these regulators in the regulation of lipopeptides and does this active regulation significantly increase the production of lipopeptides as their concentration increase? *Spo0A* , which controlled the initiation of spore formation, encoded a protein called Spo0A that not only promoted sporulation, but also was related to the synthesis of antimicrobial substances. Previous studies revealed that the regulation of iturin A was carried out by Spo0A (Rahman, Ano, & Shoda, 2006). Moreover, surfactin, as a signaling molecule, can increase the phosphorylation of Spo0A by the kinase KinC. Then, Spo0A-P will activate cannibalism, sporulation, and biofilm formation (López & Kolter, 2010). Given that they are all antimicrobial lipopeptides, it would be not surprising that*spo0A* regulates the expression of bacillomycin D. However, little research has been carried out to clarify the regulation mechanism of Spo0A on bacillomycin D.

In this study, the regulatory effects of these genes (*codY* ,*comA* , *degU* , and *spo0A* ) on lipopeptides were systematically investigated. Firstly, these genes in *B. amyloliquefaciens* fmbJ were deleted with the marker-free method to explore the influence of them on three lipopeptides synthesis. Then, transcriptome sequencing was used to analyze the gene differential expression profiles of mutants and wild fmbJ strains, and these genes were overexpressed for further verification.

# Materials and Methods

## Strains and Cultivation Conditions

All bacterial strains and plasmids used in this work are listed in Table 1. *B. amyloliquefaciens* fmbJ (CGMCC No. 0943), *Escherichia coli* DH5α, and *E. coli* JM110 were grown in Luria-Bertani (LB) medium at 37°C for strain construction. The strain was carried out in a beef extract medium (3% beef extract, 10% peptone, 5% NaCl, and pH 7.2) and cultivated at 37°C with 180 rpm as seed culture. For lipopeptides (bacillomycin D, fengycin, and surfactin) production in*B. amyloliquefaciens* , the Landy medium was used as a fermentation medium consisting of the following: 20 g/L glucose, 5 g/L L-glutamic acid, 1 g/L yeast extract, 1.0 g/L $KH_2PO_4$, 0.5 g/L $MgSO_4{\cdot}7H_2O$, 0.5 g/L KCl, 5.0 mg/L $MnSO_4$, 0.16 mg/L $CuSO_4{\cdot}5H_2O$, 0.15 mg/L $FeSO_4{\cdot}7H_2O$, and pH 7.0. The fermentation was carried out at 33°C with 180 rpm for 72 h. Additionally,

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the related antibiotics (100 μg/mL ampicillin for *E. coli* , 5 μg/mL erythromycin or 5 μg/mL chloromycetin for *B.amyloliquefaciens* ) were added to the medium if necessary.

## Construction of Strains

The knockout vectors were constructed based on the thermo-sensitive plasmid pCBS (Sun, Qian, et al., 2018). The primers used for this study were presented in Table S1. Firstly, the upstream and downstream regions of *codY* were amplified with the corresponding primers. The two fragments were linked via splicing overlap extension (SOE) polymerase chain reaction (PCR) with the primers up-codY-F and down-codY-R. Then, the integration fragment was inserted into the corresponding restriction sites (*Kpn* I/*BamH* I, because *Bgl* II and *BamH* I are the same tailed enzymes) of pCBS to obtain pCBS[?]codY. The recombinant plasmid pCBS[?]codY was transferred into *B.amyloliquefaciens* fmbJ by electrical transformation technology (Zakataeva, Nikitina, Gronskiy, Romanenkov, & Livshits, 2010). The knockout strain fmbJ[?]codY was constructed according to the previously procedure reported (Zakataeva et al., 2010), including the selection of single and double exchange transformers. Finally, the knockout strain fmbJ[?]codY was validated by PCR amplification. The other three knockout strains were constructed with the same method as that for fmbJ[?]codY and were named as fmbJ[?]comA, fmbJ[?]degU, and fmbJ[?]spo0A, respectively.

The construction of expression vectors was based on pHT43 (Zhang et al., 2017). The primers used for construction of expression vectors were presented in Table S2. First,the signaling peptide portion of the pHT43 plasmid should be removed. The *Pgrac* promoter from pHT43 and the*codY* gene from *B. amyloliquefaciens* fmbJ were amplified with the primers Pgrac-F/Pgrac (codY)-R and pHT-codY-F/R. The fusion fragment of *Pgrac* and *codY* was amplified by SOE-PCR with the primers Pgrac-F and pHT-codY-R, and then cloned into pHT43 at the restriction sites *Kpn* I and *BamH* I, resulting pHT-codY. The *codY* expression plasmid was transferred into *B.amyloliquefaciens* fmbJ. The recombinant strain fmbJcodY were verified by PCR and plasmid extraction. The other three overexpression strains were constructed with the same method as that for fmbJcodY and were named as fmbJcomA, fmbJdegU, and fmbJspo0A, respectively.

## Lipopeptides Extraction and High-Performance Liquid Chromatography (HPLC) Analysis

The lipopeptide substances were extracted using the method our previously reported (Sun, Qian, et al., 2018). Briefly, the supernatant of fermentation liquid was collected after centrifugation. Then, the pH of fermentation broth supernatant was adjusted to 2 by HCl and placed at 4°C for overnight. Precipitation was collected and the substance was extracted by methanol redissolution. Subsequently, its pH was adjusted to 7.0. After high speed centrifugation (10000 g × 10 min), the crude products of lipopeptide were obtained. Then, HPLC was used to detect them quantitatively. Bacillomycin D was detected by the method based on Gong et al. (2014), while the detection methods of surfactin and fengycin were according to Wang et al. (2010).

## Transcriptomic Analysis

*B. amyloliquefaciens* fmbJ and mutant strains (fmbJ[?]codY, fmbJ[?]comA, fmbJ[?]degU, and fmbJ[?]spo0A) were cultured in fermentation medium at 33degC with 180 rpm for 12 h. Total RNA of these strains was extracted by Trizol Reagent (TranGen Biotech, Beijing, China), and the extraction method was in accordance with the instructions. The concentration of the total RNA was detected using Nanodrop 2000 (Thermo Scientific, Waltham, MA, U.S.A.), and the quality of RNA was determined by electrophoresis on a 2.0% agarose gel. Transcriptomics sequencing was carried out on a HiSeq 2500 sequencing system (Illumina) by the Beijing Genomics Institute (BGI), Shenzhen, China. The fragments per kilobase of exon per million fragments mapped (FPKM) values for gene expression were calculated, and the DESeq software was used to detect statistically significant differences in gene expression according to the following conditions: $|\log_2(\text{Fold Change})| > 1.0$ and $p$ value $< 0.05$. Functional classification of differentially expressed genes (DEGs)

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was performed by gene ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis. Furthermore, reverse transcription quantitative real-time PCR (RT-qPCR) was used to verify the results of RNA-seq. The specific procedures of RT-qPCR were carried out according to Sun et al. (2018). The primers used for RT-qPCR were listed in Table S3, and the gene of 16 s rRNA was used as the reference to normalize the expression of genes. The relative fold change of the target gene expression was evaluated by the calculation of $2^{-Ct}$. The threshold cycle (Ct) values were obtained by the Real Time PCR System software (Step One Plus, Applied Biosystems, Foster City, CA, U.S.A.).

### Statistical Analysis

One-way analysis of variance (ANOVA) in SPSS software (SPSS, version 17.0, IBM, Armonk, NY, U.S.A.) was used for statistical analysis.$P$ values were given with the results of ANOVA. The test of Duncan was performed to detect the significance below 0.05. All results were expressed with triplicate of experiment and means ± standard deviation (SD).

## Results

### Effect of Regulatory Genes Deletion on Lipopeptides Biosynthesis

To investigate the relationship of regulatory genes and lipopeptides biosynthesis, the genes *codY* , *comA* , *degU* , and*spo0A* were successfully deleted in *B. amyloliquefaciens*fmbJ by a marker-free biological technique (Fig. S1). Subsequently, the concentrations of lipopeptides were detected in mutant strains and fmbJ. As shown in Fig. 1A, bacillomycin D production was significantly reduced after those genes' knockout. The bacillomycin D concentrations of these mutants were reduced to 154.6 ± 8.4 mg/L, 20.2 ± 5.1 mg/L, 38.9 ± 9.3 mg/L, and 2.7 ± 1.9 mg/L, respectively. These results indicated that the*codY* , *comA* , *degU* , and *spo0A* genes could positively regulate the biosynthesis of bacillomycin D. *CodY* gene knockout had no significant effect on fengycin production in comparison to strain fmbJ (72.9 ± 12.2 mg/L). However, with the deletion of*comA* , *degU* , and *spo0A* genes, the yields of fengycin decreased remarkably (Fig. 1B). From Fig. 1C, the deletion of*codY* and *degU* genes increased the production of surfactin, suggesting that *codY* and *degU* negatively regulated surfactin synthesis. In contrast, after removing of the *comA* and*spo0A* genes, the productions of surfactin were remarkably reduced to 1.8 ± 0.4 mg/L and 1.7 ± 0.5 mg/L, as compared to wild strain fmbJ (5.8 ± 0.5 mg/L).

### Transcriptome Analysis of *B. amyloliquefaciens* fmbJ in Response to Regulatory Genes Deletion

In order to comprehensively analyze the global changes in gene expression after these genes deleted, comparative transcriptome analysis was used between fmbJ and mutant strains. By performing transcriptome sequencing analysis, 1533 differentially expressed genes (DEGs) were detected in mutant strains and fmbJ at $|\log_2(\text{Fold Change})| > 1.0$ and $p$ value <0.05 (Fig. S2A). Compared with fmbJ, 159, 104, 377, and 466 genes were significantly up-regulated and 156, 211, 277, and 568 genes were significantly down-regulated in the mutant strains fmbJ[?]codY, fmbJ[?]comA, fmbJ[?]degU, and fmbJ[?]spo0A, respectively (Fig. S2B). The results of principal component analysis (PCA) indicated that there was a very high correlation among different strains, and they could form clusters clearly (Fig. S2C). A GO functional classification analysis was carried out to obtain functional overview in these DEGs (Fig. S3). They are mainly involved in metabolic process and catalytic activity, mostly from protein-coding genes located on cell membranes. Overall, the distribution of DEGs involved in biological process, cellular component and molecular function of different mutants was similar and relatively concentrated. These DEGs were further classified by KEGG pathway, as shown in Table 2. In cellular processes, the DEGs were mainly involved in cellular community and cell

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motility. Signal transduction and membrane transport were the concentrated embodiment of DEGs in environmental information processing pathway. In fmbJ[?]degU and fmbJ[?]spo0A, the DEGs related to genetic information processing were involved in translation, replication and repair, and fold, sorting and degradation. In addition, carbohydrate metabolism, amino acid metabolism, and energy metabolism were the main metabolic processes related to them. Next, the most enriched pathways of DEGs in each mutant strain were analyzed, as shown in the Fig. 2.

### *Metabolic pathway analysis of DEGs in fmbJ[?]codY*

By classifying the pathway of 315 DEGs, we found that the metabolic types of *codY* involved in transcriptional regulation in fmbJ mainly included the following aspects (Fig. 2A). In amino acid metabolism, the up-regulation of DGEs was found in the synthesis of valine, leucine and isoleucine (*leuA* , *leuB* , *leuC* ,*ilvB* , *ilvC* , *ilvH* and *ilvE* ), phenylalanine, tyrosine and tryptophan biosynthesis (*aroA* , *tyrA* , and*trpA-F* ), histidine metabolism (*hisA-C* , *hisF* ,*hisH* , *hisI* and *yfmT* ), and lysine synthesis (*yclM* and *lysC* ). In the biosynthesis of secondary metabolites, up-regulated genes were significantly more than down-regulated genes, indicating that *codY* inhibited the expression of most genes related to the synthesis of secondary metabolites. In addition, *codY* also regulated the expression of various ABC transporters, the phosphotransferase system and the two-component system. Furthermore, as Fig. 3 showed, six genes (*gltA* , *gltB* , *serA* , *tyrA* , *thrB* and*thrC* ) involved in the biosynthesis of amino acids related to bacillomycin D synthesis were up-regulated, while the genes (*ansZ* , *glmS* , and *glnA* ) associated with their catabolism were down-regulated. All the bacillomycin D synthase genes (*bmyA* , *bmyB* , *bmyC* and *bmyD* ) were down regulated. These results indicated that CodY was involved in regulating many metabolic pathways of cells, especially the metabolism of amino acids, as well as the transcription of genes related to the secondary metabolites.

### *Metabolic pathway analysis of DEGs in fmbJ[?]comA*

From Fig. 2B, after knockout of *comA* in fmbJ, most of the DEGs were down regulated in the most enriched pathways of DEGs, indicating that the deletion of *comA* weakened the overall metabolic capacity of the strain. The pathways related to *comA* in *B. amyloliquefaciens* mainly concentrated in the two-component system, quorum sensing, ABC transporters and biosynthesis of secondary metabolites. With the knockout of *comA* , the genes *comQ*(-2.41), *comP* (-1.90), *rapA* (-4.53), *rapC* (-3.73),*rapF* (-5.20), *rapH* (-4.23), and *degU* (-2.66) in the quorum sensing system were significantly down-regulated, suggesting that signal regulator ComA played an important role in the quorum sensing system. Genes involved in the transport of monosaccharides, oligosaccharides and polyhydric alcohols were down regulated. However, four oligopeptide transporters and related genes (*oppA* ,*oppB* , *oppC* , and *oppD* ) were upregulated. And,*pstA* , *pstB* , *pstC* , and *pstS* genes were significantly upregulated. Moreover, *comA* was associated with genes related to the formation of non-ribosomal peptide structure, the genes related to the synthesis of bacillomycin D, surfactin and fengycin were all down regulated. In addition, we found that nitrogen metabolism, flagellum assembly and bacterial chemotaxis were remarkably regulated by*comA* gene.

### *Metabolic pathway analysis of DEGs in fmbJ[?]degU*

In the mutant fmbJ[?]degU (Fig. 2C), the *comQ* (-1.25) and*comP* (-1.02) genes in the quorum sensing system were significantly down-regulated. The knockout of *degU* could affect the transport of substances, which was manifested in the enhanced transport capacity of phosphate and amino acids (*pstA* ,*pstB* , *pstC* , *pstS* , and *glnM* , up-regulated), the weakened transport capacity of monosaccharides, oligosaccharides and polyols, and metal ions, especially zinc ions (*znuA* , -3.70, and*znuC* , -3.29). Meantime, the deletion of *degU* also affected the synthesis of non-ribosomal peptides, in which bacillomycin D and fengycin synthase genes were down-regulated, while, surfactin synthase genes were up-regulated. Furthermore, the knockout of *degU*resulted in upregulated of genes related to mismatch repair, DNA replication, and nucleotide excision repair, while the ability to form ribosomal proteins was weakened.

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***Metabolic pathway analysis of DEGs in fmbJ[?]spo0A***

When *spo0A* gene was deleted, 54 DEGs were involved in amino acid biosynthesis, among which valine, leucine, and isoleucine biosynthesis related DEGs were upregulated. There were 87 DEGs associated with antibiotic biosynthesis, showing slightly more upregulated genes. Then, in the TCA cycle and oxidative phosphorylation, the down-regulated genes were significantly more than up-regulated ones (Fig. S4). In addition,*comA* , *comK* , *rapA* , *rapC* , *rapG* ,*rapH* , and *degU* in the quorum sensing system were down regulated. Furthermore, *spo0A* also played a key role in the synthesis of non-ribosomal peptides. Its mutation caused a whole down regulated in synthase genes of bacillomycin D, fengycin and surfactin. These results showed that Spo0A was involved in the regulation of various metabolic pathways, including central metabolism and energy metabolism, as well as the transcription of genes related to secondary metabolites (Fig. 2D).

## Effect of Regulatory Proteins on Bacillomycin D, Fengycin and Surfactin Synthase Genes

Analysis of transcriptome sequencing results of the mutants revealed that the deletion of these signaling genes not only affected bacillomycin D synthase genes expression, but also affected fengycin and surfactin synthase genes. Moreover, there were different effects on the three lipopeptide synthase genes. As shown in Fig. 4, there was a good consistency between the gene expression data analyzed by RT-qPCR and the expression level obtained by transcriptome sequencing, which fully indicated that the transcriptome sequencing results were highly reliable. It was found that the deletion genes of *codY* and*degU* resulted in the up-regulation the expression of*srfAA-srfAD* , and down-regulation the expression of*fenA-fenE* and *bmyA-bmyD* . And, they had a more significant regulatory effect on *bmyA-D* (Fig. 4A, C). The *comA* gene knockout significantly down regulated the synthase genes of the three lipopeptides. And, its regulatory effect on *srfAA-srfAD* was more obvious, with the maximum expression fold change ($\log_2$) of -4.8 (Fig. 4B). In addition, with the deletion of *spo0A* , the three lipopeptides synthase genes were significantly down-regulated, among which *bmyA-bmyD* genes were the most significantly down-regulated with expression fold change ($\log_2$) of -7.58, -8.45, -6.11, and -8.27, respectively (Fig. 4D).

## Overexpression Verified the Effect of Regulatory Genes on Bacillomycin D Biosynthesis

To further verify the role of these genes in the regulation of bacillomycin D biosynthesis, overexpressed strains fmbJcodY, fmbJcomA, fmbJdegU, and fmbJspo0A were constructed. Does bacillomycin D yield increase with the accumulation of proteins concentrations? Therefore, different concentrations of inducer IPTG were used to induce the expression of these genes. Fig. 5 showed the effects of *codY* ,*comA* , *degU* , and *spo0A* genes overexpression in fmbJ on bacillomycin D production.

As seen in Fig. 5A, the yield of bacillomycin D increased to a certain extent with the extension of fermentation time. When no inducer IPTG was added, the production of bacillomycin D reached 240.9 +- 11.8 mg/L after 72 h fermentation in the overexpressed strain fmbJcodY, which was equivalent to that of wild strain fmbJ under the same fermentation conditions. When 50 mg/L IPTG was added, the bacillomycin D production of fmbJcodY was only 40.5 +- 11.8 mg/L after 24 h. While with the increase of cultivate time, the yield of bacillomycin D was 312.4 +- 15.9 mg/L after 72 h, which was significantly higher than that of the group without IPTG. However, as the concentration of IPTG increased, the bacillomycin D production decreased instead of increasing. These results indicated that *codY* , as a global regulatory gene, could play a positive regulatory role in the synthesis of bacillomycin D in fmbJ only to a certain extent. With the accumulation of signal factor codY, the amino acid metabolism and fatty acid metabolism were controlled by inhibiting the synthesis of amino acid, thus affecting the bacillomycin D biosynthesis.

In the strain overexpressed *comA* , the production of bacillomycin D was inhibited with the addition of

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high concentration of IPTG (Fig. 5B). With the IPTG concentration increasing, the production of bacillomycin D in fmbJdegU showed an increasing trend, but there was no significant difference in the increase of bacillomycin D production caused by the IPTG concentration (Fig. 5C). The yield of bacillomycin D in strain overexpressing *spo0A* increased significantly with increasing of IPTG concentration. However, when the inducer reached 200 mg/L, there was no significantly increase in the bacillomycin D production with respect to the addition of 100 mg/L IPTG. And, its maximum production was 648.9 +- 60.9 mg/L, which was 2.3 times that of the same condition without the addition of IPTG. These results showed that *comA* might play an indirect regulatory effect on bacillomycin D synthesis, while *degU* and *spo0A* could play a direct regulatory effect on the synthesis of bacillomycin D, which might directly affect the regulatory region of the promoter of bacillomycin D synthase genes.

### Overexpression Verified the Effect of Regulatory Genes on Fengycin and Surfactin Biosynthesis

To further investigate the influence of these genes' overexpression on the biosynthesis of fengycin and surfactin, the yields of them were determined. As shown in Fig. 6A, *CodY* gene overexpression had no significant influence on the yield of fengycin in comparison to fmbJ. While, the fengycin productions of fmbJcomA (200.8 +- 8.8 mg/L), fmbJdegU (279.9 +- 2.2 mg/L), and fmbJspo0A (245.0 +- 5.6 mg/L) increased by 2.6, 3.7, and 3.2 times compared with strain fmbJ. From Fig. 6B, the overexpression of *codY* and *degU* genes reduced surfactin production, suggesting that *codY* and *degU* negatively regulated surfactin synthesis. In contrast, surfactin productions were increased to 1.4 and 1.7 times that of fmbJ through the overexpression of *comA* and *spo0A* genes.

## DISCUSSION

As a global negative regulator, CodY is usually a transcriptional repressor of its target genes, which binds to the promoter of these genes or directly to genes to block the binding of RNA polymerase (Kim, Jung, & Chai, 2016). From Fig. 2A, the DEGs in the mutant fmbJ[?]codY participated in the most enriched pathways, most of which were upregulated, that is, CodY inhibited the expression of these genes. This suggested that CodY was indeed a global transcriptional suppressor. However, our results showed that the yield of bacillomycin D produced by fmbJ[?]codY was significantly reduced, indicating that the *codY* gene could positively regulate the biosynthesis of bacillomycin D. Moreover, transcriptome sequencing results also confirmed that CodY activated the bacillomycin D synthase genes *bmyA* (-2.22),*bmyB* (-1.93), *bmyC* (-1.83), and *bmyD* (-1.93). It is likely that CodY plays an active regulatory role in the growth of bacteria, but the role is overshadowed by the negative regulatory role defined by CodY. In recent years, it had been reported that global regulatory factor CodY played a positive role in the regulation of *B. subtilis ackA* (Shivers, Dineen, & Sonenshein, 2006).

In addition, we found that CodY inhibited the expression of surfactin synthase genes, and the production of surfactin increased when it was deleted, which was consistent with the results of Wu et al. (2019), who found that the surfactin production in *B. subtilis* 168 increased after knockout of *codY* gene. Bacillomycin D and surfactin are lipopeptides, why is the regulation of CodY on surfactin and bacillomycin D different? Previous studies have shown that CodY played a direct role on the promoter region of the surfactin synthase gene *srfA* and regulated the synthesis of surfactin (Serror & Sonenshein, 1996; Stein, 2005). The regulation of CodY on bacillomycin D included its synthase genes and required amino acid metabolism. When overexpressing *codY* gene, the production of bacillomycin D increased with the expression of *codY* , but with the further accumulation of CodY, bacillomycin D production did not increase more. The results showed that the concentration level of CodY determined the mode of its regulation in the synthesis of bacillomycin D. The global regulatory factor CodY controls the expression of many genes in the late logarithmic phase and the early stationary phase in many gram-positive bacteria (Sonenshein, 2005). In *B. subtilis* , the interaction of CodY and two cofactors (GTP and branched chain amino acids) enhanced CodY and DNA binding activity

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(Molle et al., 2003). Excessive accumulation of branched chain amino acids would lead to inhibition of nitrogen catabolism and reduction of secondary metabolites production (Cai et al., 2019). Bacillomycin D was a cyclic lipopeptide composed of fatty acid chain and seven amino acid (L-Asn, D-Tyr, D-Asn, L-Pro, L-Glu, D-Ser, and L-Thr) forming a peptide chain (Peypoux et al., 1984). Although CodY could activate the bacillomycin D synthase genes, it could also inhibit the biosynthesis of amino acids required by bacillomycin D. For example, transcriptome sequencing data showed that the differential genes *gltA* and *gltB* were up-regulated, while, *glmS*and *glnA* were down-regulated in mutant strain fmbJ[?]codY. This indicated that CodY inhibited the biosynthesis of glutamate. If the accumulation of CodY was more, the biosynthesis of amino acids required by bacillomycin D would be inhibited, and the reason of the concentration dependence of the global regulatory factor CodY on the regulation of bacillomycin D would also be explained.

ComA and DegU, as signal regulators in the two-component system, control many processes of cells, including biofilm and competence formation, bacterial motility, and antibacterial substances synthesis (Hamoen, Venema, & Kuipers, 2003; Harshey, 2003; Kobayashi, 2007; Koumoutsi et al., 2007; Mariappan, Makarewicz, Chen, & Borriss, 2012; Verhamme, Kiley, & Stanley-Wall, 2007; P. Wang et al., 2015; Zhang et al., 2017). As an important protein controlling spore formation, Spo0A played a key regulation role in the synthesis of iturin A and bacilysin (Boguslawski, Hill, & Griffith, 2015; Rahman et al., 2006; Yazgan Karatadeg, Cetin, & Ozcengiz, 2003). Our previous researches showed that bacillomycin D synthesis was related to *comA* , *degU* , and *spo0A*(Qian et al., 2015; Qian et al., 2017). In the study, the deletion strains showed the productions of bacillomycin D were remarkably lower than that of wild strain fmbJ.

Knockout of *comA* gene could significantly reduce the production of three lipopeptides. Meantime, its overexpression could also improve the lipopeptides production. Nonetheless, the results indicated that with the increase of ComA concentration, the yield of bacillomycin D was inhibited. Phosphorylated ComA (ComA-P) played a role in the synthesis of antimicrobial lipopeptides. For example, during the synthesis of surfactin, ComA-P directly combined to the *srfA* promoter of surfactin synthase genes and played a regulatory role (Lopez & Kolter, 2010; Nakano, Xia, & Zuber, 1991; Yang et al., 2015). The results of transcriptome sequencing showed that after the knockout of *comA* ,*degU* gene was significantly down-regulated (-2.66). It was concluded that ComA might indirectly regulate bacillomycin D synthesis through DegU. Meanwhile, from ComA to ComA-P, ComP was needed to provide phosphate group. Mass accumulation of ComA resulted in energy consumption, and the amount of ComP was not enough to supply ComA-P, which might be the reason why the accumulation of ComA caused the inhibition of the yield of bacillomycin D. Interestingly, when the*comA* gene was integrated into the fmbJ genome, we found a significant increase in bacillomycin D production (data unpublished).

The deletion of *degU* gene reduced the yields of bacillomycin D and fengycin, but increased surfactin production. Mader et al. (2002) reported that DegU repressed the *srf* operon and therefore inhibited the synthesis of surfactin. This is consistent with our results. With the overexpression of *degU* , bacillomycin D production was increased, but not significantly. Generally, DegU has two modes of effect: the phosphorylated DegU (DegU-P) directly activates the production of degradation enzyme and restricted motility, while unphosphorylated DegU by combined with *comK* promoter directly stimulates the formation of competence (Dahl, Msadek, Kunst, & Rapoport, 1992; Hamoen et al., 2003). Although DegU directly acts on the promoter region of bacillomycin D synthase (Koumoutsi et al., 2007), it is possible that the unphosphorylated DegU has a weak binding ability to target DNA, and it is the DegU-P form that really plays a role in the regulation of bacillomycin D synthesis.

The deletion and overexpression of *spo0A* gene could markedly reduce or increase the productions of lipopeptides. Furthermore, the yields of bacillomycin D were remarkably improved with increasing of Spo0A concentration. Transcriptome sequencing of the mutant strain fmbJ[?]spo0A showed that *abrB* gene was up-regulated (1.82),*comA* and *degU* genes were respectively down-regulated -1.14 and -2.69. These results suggested that Spo0A played a vital part in the lipopeptides synthesis. Generally, the response regulator Spo0A controls the sporulation pathway (Widderich et al., 2016). Meanwhile, it is also closely related to the formation of biofilms (Park, Hussain, Wei, Kwon, & Oh, 2019). The relationship between Spo0A and

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lipopeptides is likely to regulate lipopeptides biosynthesis by the mechanisms of spore and biofilm formation. Surfactin is a powerful surfactant that acts on cell membranes and destroys their integrity. As a signaling molecule, surfactin selectively induces potassium leakage and promotes biofilm formation on the basis of membrane disturbance and KinC perception (Lopez, Fischbach, Chu, Losick, & Kolter, 2009). While, the sensor kinases KinA-E activate the regulator Spo0A through phosphorylation, which in turn triggers the sporulation and biofilm formation (Aleti et al., 2016). Therefore, the production of lipopeptides has a complex relationship with biofilm and spore formation. Spo0A may also exist in two modes in the regulation of lipopeptides synthesis: the phosphorylated Spo0A (Spo0A-P) activates the sporulation and biofilm formation, while unphosphorylated Spo0A may act directly on the regulator region of lipopeptides synthase genes. However, the real mechanism of Spo0A in lipopeptides synthesis is unknown. In Fig. 7, the regulation model of these signaling genes to antimicrobial lipopeptides in *B. amyloliquefaciens* is presented.

In conclusion, genes knockout, overexpression and transcriptome sequencing have shown that the genes (*codY* , *comA* ,*degU* and *spo0A* ) played an important role in the biosynthesis of lipopeptides. They positively regulated the expression of bacillomycin D and fengycin synthase genes in strain fmbJ. However, the genes *codY* and *degU* negatively regulated surfactin biosynthesis. Moreover, it was the first time to report the concentration dependence of the global regulatory factor CodY on the synthesis of bacillomycin D. And, Spo0A might play a direct regulatory role in the synthesis and secretion of bacillomycin D. But, the exact mechanism of Spo0A in lipopeptides biosynthesis remains to be determined.

## Data Availability

The transcriptome sequencing data of *B. amyloliquefaciens* fmbJ, fmbJ[?]codY, fmbJ[?]comA, fmbJ[?]degU, and fmbJ[?]spo0A are available in the SRA database under submission number SUB7440200 and BioProject accession number PRJNA635166.

**Reviewer link as follows:**

https://dataview.ncbi.nlm.nih.gov/object/PRJNA635166?reviewer=9qh928kvt4gk0a4slbmutufb5b

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Authors' Contributions

Jing Sun, Zhaoxin Lu, and Yingjian Lu conceived and designed research. Jing Sun and Yanan Liu conducted experiments. Jing Sun and Fuxing Lin analyzed data. Jing Sun wrote the manuscript. All authors read and approved the manuscript.

Ethical Statement

## *Conflicts of interest*

The authors declare that they have no conflict of interest.

## *Ethical approval*

This article does not contain any studies with humans or animals performed by any of the authors.

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**Figure Captions**

**Fig. 1** Influence of the regulatory genes' deletion on the productions of bacillomycin D (A), fengycin (B) and surfactin (C) in*B. amyloliquefaciens* fmbJ. The strains fmbJ and mutants were cultured in 50 mL of Landy medium at 33degC with 180 rpm for 72 h. * and ** indicated the significant differences from control at $p< 0.05$ and $p < 0.01$, respectively.

**Fig. 2**The most enriched pathway of differentially expressed genes in four mutant strains and fmbJ. A-D represent the mutant strains fmbJ[?]codY, fmbJ[?]comA, fmbJ[?]degU, and fmbJ[?]spo0A, respectively.

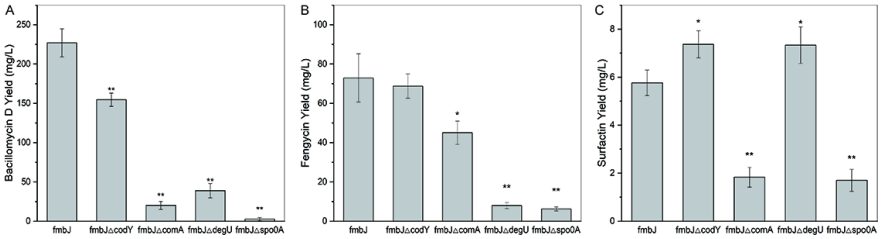
**Fig. 3** Metabolic pathway network for bacillomycin D biosynthesis in mutant strain fmbJ[?]codY based on KEGG pathway analysis. The red labelled genes indicate up-regulated, the green labelled genes, down-regulated. The gray boxes represent bacillomycin D and related amino acids. The scissor means to knock out *codY* gene.

**Fig. 4** Influence of the regulatory genes' deletion on bacillomycin D, fengycin, and surfactin synthase genes. A-D represented the effect of knockout genes *codY* , *comA* , *degU* , and*spo0A* on these synthase genes, respectively.

**Fig. 5** Influence of *codY* , *comA* , *degU* , and*spo0A* gene overexpression on bacillomycin D production in*B. amyloliquefaciens* fmbJ. Keys: A, fmbJcodY; B, fmbJcomA; C, fmbJdegU; D, fmbJspo0A. The strains were cultured in 50 mL of Landy medium at 33degC with 180 rpm for 24 h, 36 h, 48 h, 60 h, and 72 h.

**Fig. 6** Influence of the regulatory proteins' genes overexpression on the productions of fengycin (A) and surfactin (B) in *B. amyloliquefaciens* fmbJ. The strains were cultured in 50 mL of Landy medium at 33degC with 180 rpm for 72 h. * and ** indicated the significant differences from control at $p< 0.05$ and $p < 0.01$, respectively.

**Fig. 7** The regulation model of regulatory proteins to three lipopeptides in *B. amyloliquefaciens* .



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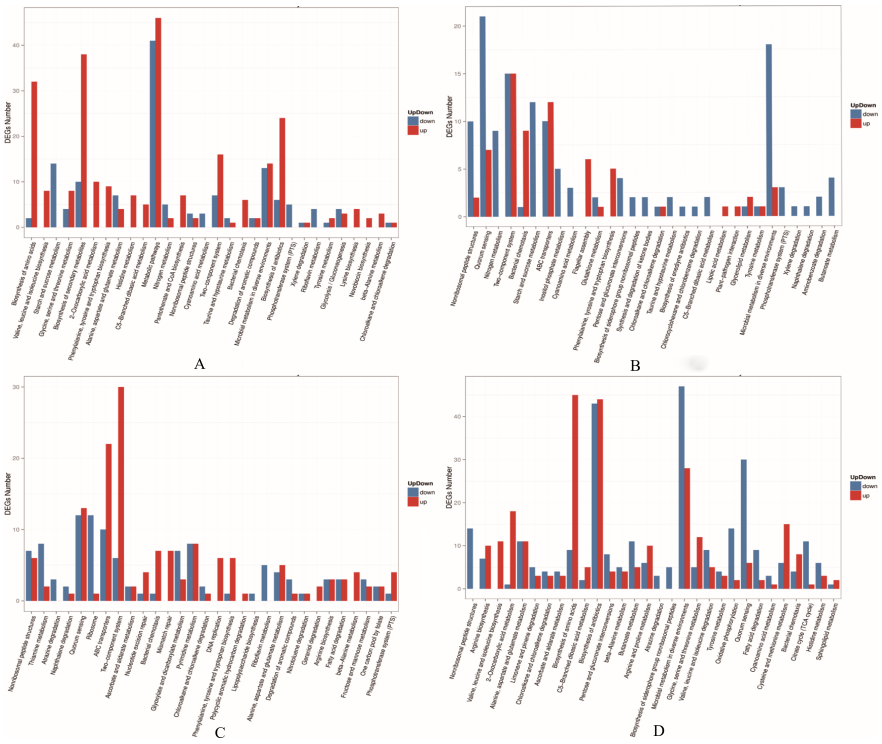

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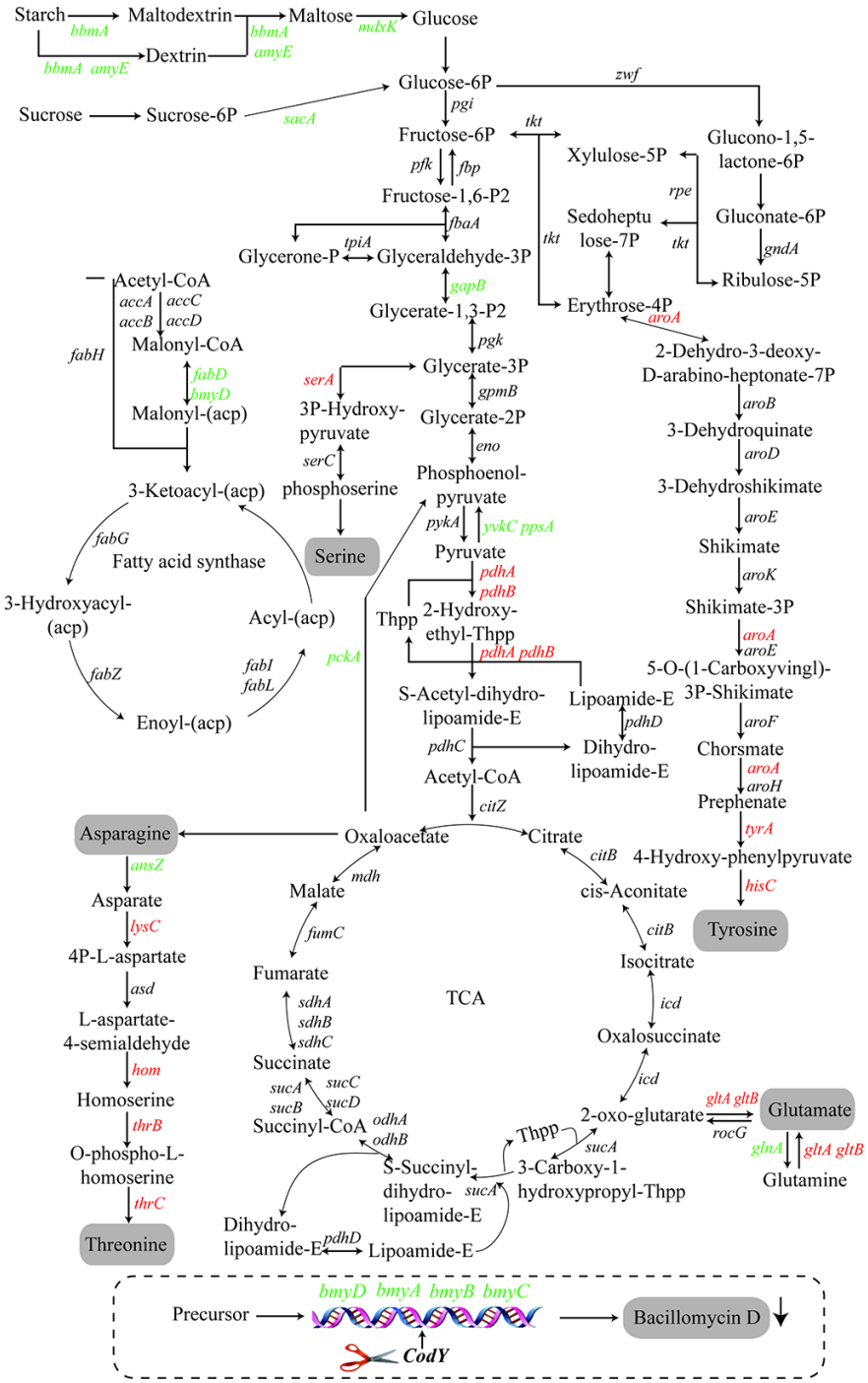

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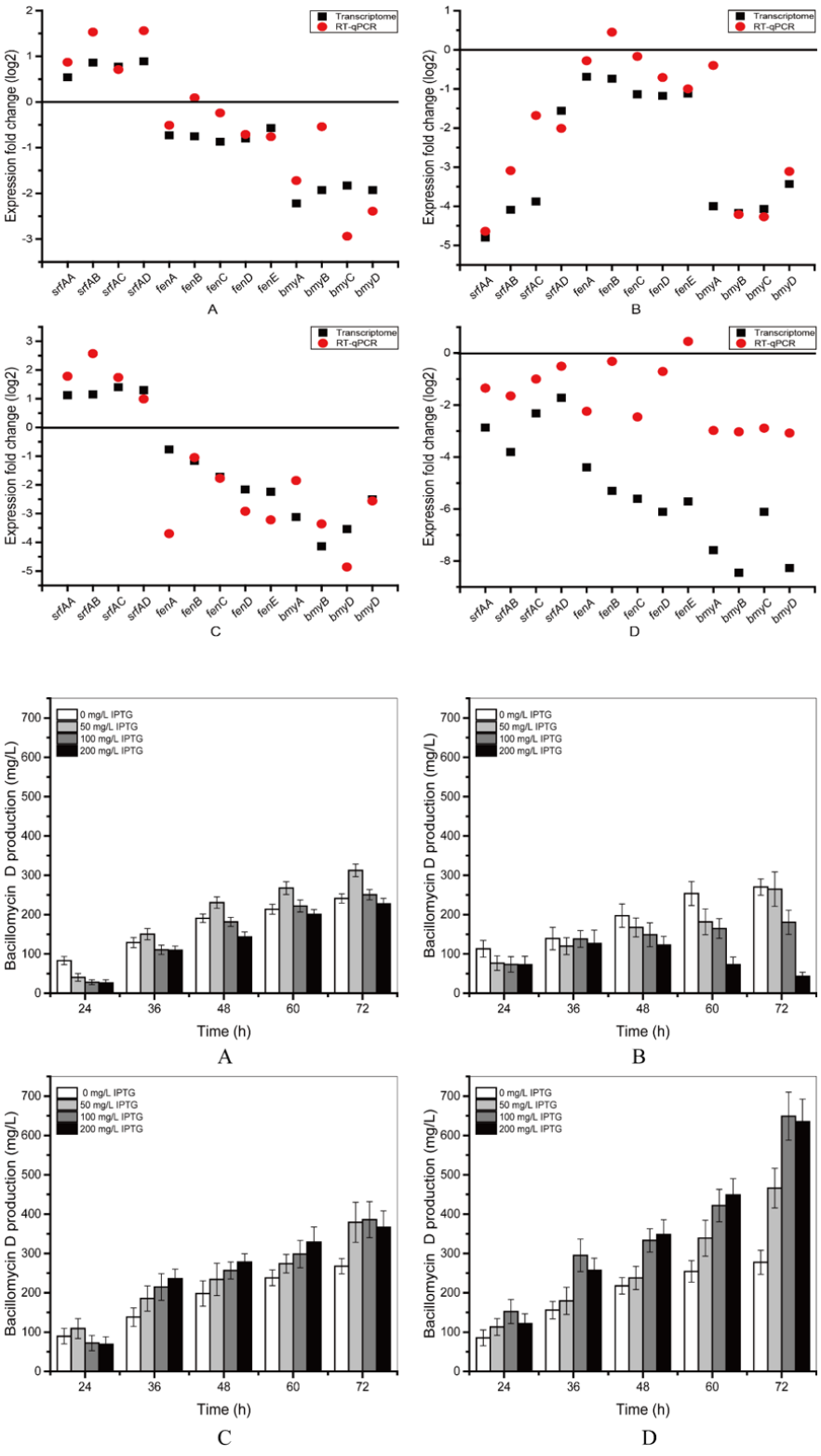

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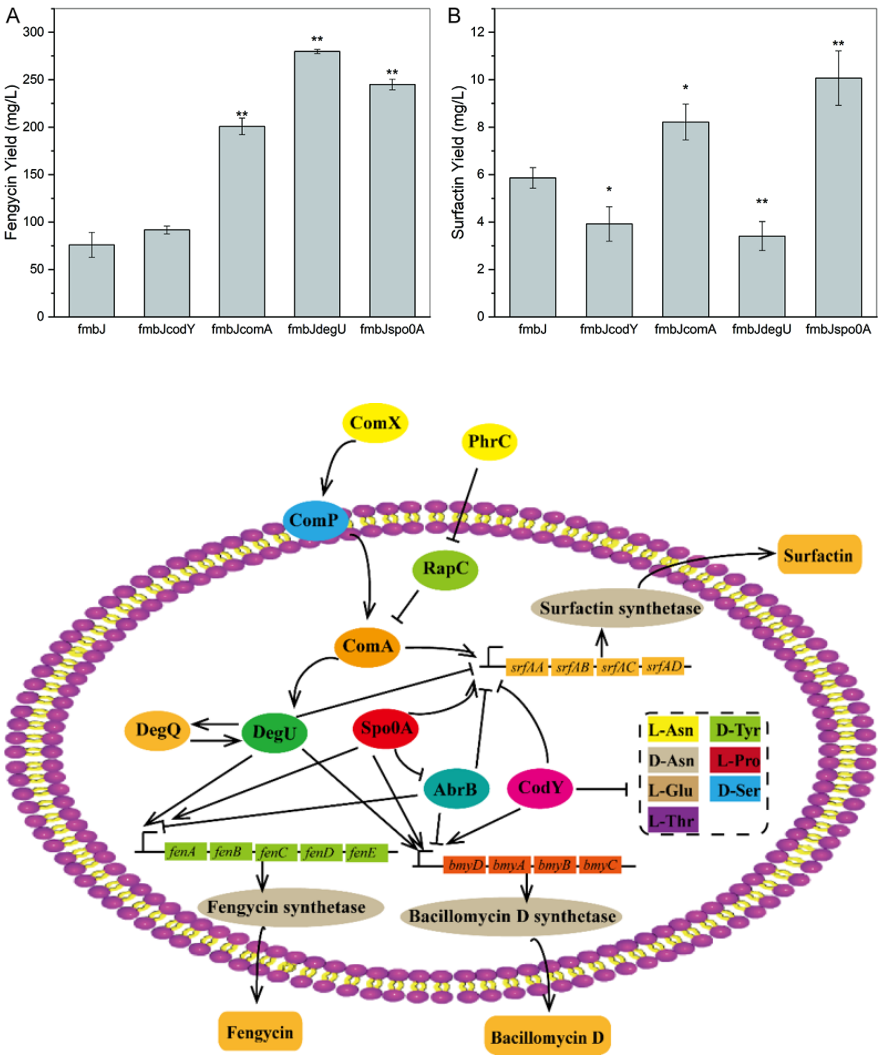


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