Manipulation of Purine Metabolic Networks for Riboflavin Production in *Bacillus subtilis*

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ABSTRACT: Guanosine monophosphate, the precursor for riboflavin biosynthesis, can be converted to or generated from other purine compounds in purine metabolic networks. In this study, genes in these networks were manipulated in a riboflavin producer, *Bacillus subtilis* R, to test their contribution to riboflavin biosynthesis. Knocking out adenine phosphoribosyltransferase (*apt*), xanthine phosphoribosyltransferase (*xpt*), and adenine deaminase (*adeC*) increased the riboflavin production by 14.02, 6.78, and 41.50%, respectively, while other deletions in the salvage pathway, interconversion pathway, and nucleoside decomposition genes have no positive effects. The enhancement of riboflavin production in *apt* and *adeC* deletion mutants is dependent on the purine biosynthesis regulator PurR. Repression of ribonucleotide reductases (RNRs) led to a 13.12% increase of riboflavin production, which also increased in two RNR regulator mutants PerR and NrdR by 37.52 and 8.09%, respectively. The generation of deoxyribonucleoside competed for precursors with riboflavin biosynthesis, while other pathways do not contribute to the supply of precursors; nevertheless, they have regulatory effects.

INTRODUCTION

Riboflavin, also called vitamin B2, is mainly used as an animal feed additive and a dietary supplement. Industrial production of riboflavin via fermentation has been developed for decades, and there have been many studies on the development of riboflavin-producing strains. Among them, the most detailed studies involved strategies for engineering the central carbon metabolism and purine biosynthesis pathway. Overall, the studies concluded that the supply of the two precursors ribulose-5-phosphate (Ru5P) and guanosine triphosphate (GTP) is crucial for riboflavin biosynthesis. GTP is mainly produced by the purine de novo biosynthesis pathway, where the carbon flow can be increased by overexpressing the genes in the purine operon. In addition, other strategies such as depression of the regulator and riboswitch are applied in engineering the de novo synthesis pathways.

GTP is mainly produced by the purine de novo biosynthesis pathway, but it can also be synthesized through the salvage pathway from purine bases or purine nucleosides. These reactions were catalyzed by purine phosphoribosyltransferases, including adenine phosphoribosyltransferase (*apt*), hypoxanthine–guanine phosphoribosyltransferase (*hptT*), and xanthine phosphoribosyltransferase (*xpt*). *Bacillus subtilis* and other riboflavin producers are also suitable for nucleoside production, and early reports indicated that salvage pathways can be adopted for nucleoside biosynthesis.

Moreover, *B. subtilis* can also produce nucleosides via the dephosphorylation of nucleotides, and in recent reports, enzymes with corresponding activities have been identified. *YktC* had 5′-nucleotidase activity, where guanosine 5′-monophosphate (GMP) and inosine 5′-monophosphate (IMP) are the preferential substrates, while YcsE exhibited strong 5′-nucleotidase activity with a wider specificity. Furthermore, reactions opposite to the salvage synthesis of purine nucleosides are also present in *B. subtilis*, enabling it to decompose nucleosides into purine bases. In *B. subtilis*, *deoD* and *pupG* are encoded purine nucleoside phosphorylases, which catalyzed the decomposition reactions. *adeC* encoded the adenine deaminase, *guaD* encoded the guanine deaminase, and *pupABCD* encoded the xanthine dehydrogenase, which catalyzed the interconversion of purine bases. Finally, purine ribonucleotide can also be transformed into a deoxyribonucleotide, which is catalyzed by ribonucleotide reductases (RNRs). Together with the de novo synthesis pathway, these pathways constitute the purine metabolism.

Received: August 12, 2020  
Accepted: October 6, 2020  
Published: November 2, 2020
network (Figure 1). However, it remains unknown whether these reactions influence the supply of precursors for riboflavin biosynthesis.

In this study, genes encoding enzymes of the purine salvage pathway, interconversion pathway, nucleotidases, and RNRs were knocked out and/or expressed in the riboflavin-producing strain B. subtilis R to assess their contribution to riboflavin biosynthesis. In doing so, we clarified the real effects of these manipulations on riboflavin production, revealing that the deoxyribonucleoside triphosphate (dNTP) synthesis pathway competes for precursors of riboflavin biosynthesis, while the adenine metabolism affects the production of riboflavin, in which the purine synthesis regulator is involved.
Effects of the Manipulation of the Purine Salvage Pathway and Interconversion Pathway on Riboflavin Production. We chose purine phosphoribosyltransferase genes (apt, xpt, and hprT), purine interconversion genes (guaD, pucABCDE, and adeC), purine nucleoside phosphor- ylase genes (adeD and pugG), nucleotidase genes (yjKN, yunD, ykC, and ypsE), and RNR genes (nrdE and nrdF) as candidates for manipulating the purine metabolic network (Figure 1). We first investigated the effects of the deletion mutations of the purine salvage pathway and interconversion pathway genes on the production of riboflavin. To test the effect of these genes on the production of riboflavin, a riboflavin producer with a basic level of production was constructed. For genome manipulation, the chromosome araR of B. subtilis 168 was replaced with the promoter of araR and neo (the neomycin resistance gene). First, the regulation region of the rib operon is engineered to deregulate the expression of the rib operon, generating BSYYM. Subsequently, a mutation of adenylsulfamide synthetase (purA) and a mutation of ribulose phosphate epimerase (rpe) (unpublished work) were introduced into the chromosome to reduce the carbon flow to the byproducts, generating BSLY and BSR separately. Then, the mutants were constructed based on BSR, and all the target genes except guanine deaminase (guaD) were deleted successfully. According to the SubtiWiki database (subtiwiki.uni-goettingen.de), guaD is a nonessential gene, and it could be knocked out in the parent strain—BSLY (rpe), but we failed to obtain positive clones after transforming BSR with the knockout fragment. Similarly, GMP reductase (encoded by gca), which is a part of a pathway for converting GMP back to IMP, could not be knocked out on the background of BSR1 (rpe*1) and could be knocked out in BSLY (rpe*2). This indicated that BSR had a changed metabolism of purines, especially for guanine compared with BSLY, causing gud and gca to become essential.

In shake-flask fermentations, the highest improvement of riboflavin titer (41.50%) was observed in strain BSR4 (ΔadeC), followed by BSR1 (Δapt) (14.02%), while BSR2 (Δxpt) only showed a small increase (6.78%) at 42 h (Figure 2a). Different from adenine deaminase (adeC), which generates hypoxanthine from adenine, xanthine dehydrogenase (pucABCDE) converts hypoxanthine into xanthine. Moreover, BSR5 (ΔpucABCDE) had a lower riboflavin titer than BSR (a decrease by 12.63%) (Figure 2a). It is noteworthy that the dry cell weight (DCW) of BSR4 (ΔadeC) was decreased by 16.40% compared to that of BSR (Figure 2a), resulting in a 67.86% enhancement of productivity at 42 h, while the DCW of BSR3 (ΔhprT) decreased by 13.93% (Figure 2a), and the productivity increased by 29.26%. The DCW of BSR5 (ΔpucABCDE) decreased by 16.57% (Figure 2a), and the productivity is similar to that of BSR. Moreover, we also combined the deletion of adeC and apt to construct BSR19. However, BSR19 produced 306.31 mg/L riboflavin in 42 h fermentation, which is similar to that of BSR2 (Δapt) (Figure S1a).

The abovementioned results showed that none of the deletions of the salvage pathway genes cause a remarkable decrease of riboflavin production. Then, we constructed the plasmids expressing xpt and hprT and introduced them into BSR to generate BSR/ppxpt and BSR/phpT, respectively. The fermentation results indicated that the expression of xpt and hprT has no positive effect on riboflavin production (Figure S2a). Therefore, we confirmed that the salvage pathway is not used for GMP synthesis in riboflavin production (at least in our fermentation medium, which only contained corn steep powder, yeast extract, and (NH₄)₂SO₄ as nitrogen sources without an additional purine source). The plasmids expressing apt, adeC, and pucABCDE were also constructed and introduced into BSR to generate BSR/papt, BSR/padeC, and BSR/ppucABCDE. The riboflavin production of BSR/papt is 5.40% lower than that of BSR12, while the riboflavin production of BSR/padeC is similar to that of BSR12. Moreover, the production of BSR/ppucABCDE is 9.89% higher than that of BSR12.

To investigate the roles of guaC and guaD in riboflavin production, expression plasmids were constructed and introduced into BSR to generate BSR/pguaC and BSR/pguaD, respectively. As shown in Figure S2c, the growth of these two strains is similar to the growth of their parent strain, while the expression of the two genes decreased the titer of riboflavin by 12.75 and 12.04% compared to that of the parent strain at 42 h. The above results indicated that while the blockage of metabolic flow from guanine to hypoxanthine or xanthine compounds was detrimental to our riboflavin producer, the enhancement of these pathways has a negative effect on riboflavin production.

As the abovementioned results indicated, the salvage pathway has a limited contribution to the supply of precursors for riboflavin biosynthesis. Therefore, the variations of riboflavin production, followed by manipulating the interconversion pathway, also have nothing to do with salvage synthesis. Consequently, we wondered that other mechanisms may be involved in these results, especially the enhancement of riboflavin production in the adeC and apt deletion mutants.

Mechanism of Enhanced Riboflavin Production in the adeC and apt Deletion Strains. As discussed above, BSR1 (Δapt) and BSR4 (ΔadeC) showed the most remarkable improvement of riboflavin titer, and both genes are involved in the adenine metabolism. Previous reports mentioned the negative effect of adenine on the transcription of the purine operon. Other studies ascribed this transcriptional inhibition to the increased synthesis of adenosine S'-monophosphate (AMP) and an overflow effect of the inhibition of phosphoribosyl pyrophosphate (PRPP) synthetase, which produced less PRPP. The inhibition of PRPP by PurR is weakened, and PurR binds to the pur box in the regulatory region to decrease its transcription. We inferred that the changed transcription levels of the purR-regulated genes may have an influence on riboflavin production and that this effect is mediated by adenine. We constructed the adeC and apt deletion strains in the background of BSRP (ΔpurR) to generate BSRP1 (ΔpurR and Δapt) and BSRP2 (ΔpurR and ΔadeC). As shown in Figure 2b, the promoting effect of adeC and apt on riboflavin production disappeared, which indicated that this promoting effect is associated with PurR. To further confirm our hypothesis, adenine was added to the medium and riboflavin production was tested. The titer of riboflavin of BSR at 42 h was decreased by 10.21 and 11.58% when adenine was added at final concentrations of 25 and 35 mg/L, respectively. By contrast, the riboflavin titer of BSR1 decreased by only 2.25 and 4.18%. The titer of BSRP did not change when 25 mg/L adenine was added and decreased by 2.42% when 35 mg/L adenine was added. The titer of BSR4 did not change under both conditions (Figure 2c). These results indicated that the
apt and adeC deletion strains can “resist” a higher concentration of adenine than their parent strain, which may be caused by a change of their adenine excretion and incorporation ability.\(^{23}\) Moreover, most importantly, the involvement of PurR indicated that the adenine metabolism regulated the purine de novo synthesis pathway. In addition, the deletion of apt and adeC increased the de novo synthesis of GMP and finally promoted the riboflavin biosynthesis.

Effects of Manipulating the 5′-Nucleotidase and Purine Nucleoside Phosphorylase Genes on Riboflavin Production. For a long time, the enzymes responsible for converting nucleotides to nucleosides remained obscure even in the nucleoside-producing \textit{B. subtilis}. Recently, Terakawa and co-workers identified six enzymes in \textit{B. subtilis} that possess this activity.\(^{20}\) They found that inositol monophosphatase (encoded by yktC) exhibits phosphatase activity and plays a major role in the formation of inosine, adenosine, and guanosine. They also found that the enzyme encoded by ycsE has a significant 5′-nucleotidase activity with a broader specificity. According to KEGG pathway analysis (https://www.kegg.jp/kegg/pathway.html), the trifunctional nucleotide phosphoesterase also exhibits 5′-nucleotidase activity. We therefore tested the function of these three enzymes as well as a putative inositol monophosphatase whose gene was found to be mutated in an inosine-producing strain of \textit{B. subtilis}.\(^{26}\) As shown in Figure 3a, the riboflavin titers of the strains BSR6 (\(\Delta yfkN\)), BSR7 (\(\Delta yunD\)), BSR8 (\(\Delta yktC\)), and BSR9 (\(\Delta ycsE\)) were all lower than that of the control strain. Although BSR7 (\(\Delta yunD\)) and BSR9 (\(\Delta ycsE\)) showed an increase of the riboflavin titer at 12 h (5.68 and 8.57%, respectively), their titers decreased to the level of the control strain at 18 h (data are not shown). The deletion of these genes decreases riboflavin titers at 42 h by 8.05% (\(\Delta yfkN\)), 5.90% (\(\Delta yunD\)), 8.80% (\(\Delta yktC\)), and 10.40% (\(\Delta ycsE\)). Besides, the DCW of BSR7 (\(\Delta yunD\)) and BSR9 (\(\Delta ycsE\)) increased by 9.18% and 5.05%, respectively, while the DCW of the other two strains exhibited no changes compared with that of the parent strain. The blockage of further nucleoside degradation had a similar effect, as shown in Figure 3b, and the strains BSR10 (\(\Delta deoD\)) and BSR11 (\(\Delta pupG\)) showed a decrease of the riboflavin titer by 18.50% and 2.28% compared with that of BSR, respectively. Moreover, the DCW of BSR10 (\(\Delta deoD\)) also decreased by 9.34% compared with that of the BSR. These results indicated that the blockage of the pathways that convert nucleotides to nucleosides and further to purine bases has no benefit for riboflavin biosynthesis.

Figure 3. Influence of the manipulation of 5′-nucleotidase and ribonucleotide phosphoesterase genes on riboflavin production. (a) Comparison of riboflavin production and biomass between purine nucleoside phosphorylase gene deletion mutants and their parent strain at 42 h. (b) Ribonucleotide phosphoesterase deletion mutants and their parent strain at 42 h. The results are represented by the mean values of three replicates and the error bar by the standard deviation.

Figure 4. Effect of repression of the transcription of RNRs on riboflavin production. (a) Position of the n20 sequence of the CRISPRi inhibition site on the chromosome. (b) Comparison of riboflavin production and biomass between RNR repression strains and their parent strain at 42 h. (c) Changes of the relative transcriptional level of \textit{nrdEF} in the repression strains and their parent strain. The results are represented by the mean values of three replicates and the error bar by the standard deviation.
Effects of Manipulating NNRs on Riboflavin Production. An NNR is composed of an α-subunit and a β-subunit (encoded by \(nrde\) and \(nrdf\), respectively) and catalyzes the reduction of ribonucleoside diphosphates (NDPs) to deoxyribonucleoside diphosphates (dNDPs), providing building blocks for DNA replication. These reactions are essential for cell growth because they provide the only pathway for the biosynthesis of dNDPs and dNTPs. Unfortunately, this reaction has a large flux and competes for precursors of GDP and GTP synthesis, and we wondered whether downregulating the expression of NNRs could benefit riboflavin production. Because \(nrde\) and \(nrdf\) are essential genes in \(B.\ subtilis\), our first intention was to decrease their expression using a CRISPER interference system. The transcription of \(nrde\) and \(nrdf\) was downregulated using a series of sgRNAs, which were constructed to target protospacer adjacent motif sites with different distances from the start codon (Figure 4a). We expected an increase of riboflavin titer caused by the decrease of the expression of \(nrde\)F, but only BSRD3 produced 13% more riboflavin than the control (BSRDCas9) at 42 h (Figure 4b). However, the biomass of BSRD3 increased by 8.70%, and the productivity increased by 4.18%. Consequently, we measured the transcriptional levels of \(nrde\)F using quantitative real-time polymerase chain reaction (qRT-PCR). As shown in Figure 4c, the transcription of \(nrde\)F was only observed in BSRD3 (81.1% lower than in BSRD). Moreover, in BSRD2, the transcription level was actually enhanced 2.26-fold compared to that in BSRD. As expected, its riboflavin production decreased by 24.60% (Figure 4b).

Effects of Manipulating Nucleotide Reductase Regulators on Riboflavin Production. In \(B.\ subtilis\), the transcription of nucleotide reductase is controlled by two major regulators: PerR and NrdR. The latter regulates the expression of \(nrdl\, nrde\, nrdf\, \text{and}\, yrmB\), while PerR, a peroxide regulon, controls the expression of 25 genes, which also include \(nrdf\, nrde\, nrdf\, \text{and}\, yrmB\). Because PerR is a transcriptional repressor, the transcriptional level of \(nrdf\) in the BSR14 (ΔperR) and BSR15 (ΔnrdR) strains increased 1.88- and 21.11-fold, respectively, over the parent strain (Figure 5c). As expected, the fermentation results showed that the riboflavin titer of BSR14 (ΔperR) and BSR15 (ΔnrdR) decreased dramatically compared to that of BSR, reaching only 5.97% of the value in the parent strain BSR14 and 2.71% in BSR15 (Figure 5a).

Recently, in our lab, a mutant variant of PerR (L71V) and a mutant variant of NrdR (R26H) were identified in a riboflavin producer (data are not shown). We integrated the mutant perR and nrdR by replacing the wide-type genes in the chromosome, individually and in combination, into BSR to generate BSR16 (perR\textsuperscript{−}), BSR17 (nrdR\textsuperscript{−}), and BSR18 (perR\textsuperscript{−} and nrdR\textsuperscript{−}), respectively. Riboflavin production and the transcriptional levels of \(nrde\) and \(nrdf\) in these strains were measured. As shown in Figure 5b, the single mutants of perR and nrdR increased the riboflavin titer by 37.52 and 8.09% at 42 h, respectively, and the double mutant increased the riboflavin titer by 46.79% (Figure 5b). The biomass of BSR16 (perR\textsuperscript{−}) and BSR18 (perR\textsuperscript{−} and nrdR\textsuperscript{−}) also increased 31.1 and 35.49%, respectively (Figure 5b). Moreover, the productivity of BSR16 (perR\textsuperscript{−}) and BSR17 (nrdR\textsuperscript{−}), and BSR18 (perR\textsuperscript{−} and nrdR\textsuperscript{−}) increased 5.04, 9.34, and 10.75%, respectively. Furthermore, the expression of \(nrde\) and \(nrdf\) decreased by 31.62% (BSR16), 40.40% (BSR17), and 66.08% (BSR18) compared with that of BSR. The variation of transcriptional level and riboflavin production of \(nrdf\) was consistent with the result of BSRD3, while it was opposite to the results of BSR14 (ΔperR) and BSR15 (ΔnrdR). The increase of riboflavin productions in BSR16 (perR\textsuperscript{−}), BSR17 (nrdR\textsuperscript{−}), and BSR18 (perR\textsuperscript{−} and nrdR\textsuperscript{−}) may have a relationship with the increasing binding force of these regulators to the cis-acting element of \(nrdf\). These results confirmed that riboflavin biosynthesis can be enhanced by repressing the expression of the NNR. Therefore,
different from other pathways investigated in this study, the synthesis of dNDP is competed with precursors for riboflavin biosynthesis.

**MATERIALS AND METHODS**

**Strain, Plasmids, Media, and Chemicals.** The bacterial strains and plasmids used in this study are listed in Table S1. All *B. subtilis* strains were derived from the wild-type *B. subtilis* 168. *Escherichia coli* DH5α was used for routine transformation and maintenance of plasmids. When required, antibiotics were added to the growth media at the following concentrations: 100 μg/mL spectinomycin for *E. coli* selection and 250 μg/mL for *B. subtilis* selection, 8 μg/mL chloramphenicol for *B. subtilis* selection, and 20 μg/mL neomycin for *B. subtilis* selection. Isopropyl-beta-D-thiogalactopyranoside (IPTG) was added at a final concentration of 0.1 mM when needed.

**Construction of Strains and Plasmids.** The isolation and manipulation of recombinant DNA was performed using standard protocols. *B. subtilis* transformation was performed as described before.29 All deletion mutants were constructed using a markerless mutation delivery system.29 All primers are listed in Table S2. The medium copy number plasmid pHPl3 (spe) and the PvegI promoter were used for gene expression. The expression plasmids were constructed by Gibson assembly. The transcription interference plasmid contained an IPTG-inducible dCas9 expression cassette with the Pgrac promoter and a gRNA expression cassette with the PvegI promoter.

**Flask Fermentation and Measurement of the Riboflavin Titer.** Fermentation was performed as follows: to test the riboflavin biosynthesis ability of the constructed strains, 20 μL of a cryopreservation stock stored at −80 °C was mixed with 100 μL of sterile ddH2O, spread on an agar plate, and incubated at 37 °C for 24 h. After that, the colonies on the plate were scraped and suspended in 1 mL of the fermentation medium to acquire the seed suspension, which was used to inoculate 500 mL fermentation medium using an RNA prep Pure Cell/Bacteria Kit (Tiangen, Beijing, China) following the manufacturer instructions. Briefly, 100 ng of cDNA was used in a total reaction volume of 20 μL with 250 μM of each primer (Table S2). The fold change of each transcript in each sample relative to the control was measured in triplicate, normalized to the internal control gene (16S RNA), and calculated according to the comparative CT method.30

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c03867.

Effect of the double deletion of *apt* and *adeC* on biomass and riboflavin production; influence of expressing *guaC, guaD, xpt, hprT, adeC, apt,* and *pucABCDE* on riboflavin production; bacterial strains and plasmids used in this study; and PCR primers used in this study (PDF)

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**Notes**

The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This work was financially supported by the National Key R&D Program of China (2018YFD0901001), the Tianjin Science Fund for Distinguished Young Scholars (17JCQJC45300), the Science and Technology Service Network (STS) Initiative of the Chinese Academy of Sciences (CAS) (KF-J_STS-ZDTP-065), the Basic Research Projects of Liaoning Higher Education Institutions (2017030), and the Dalian High-level Talent Innovation Support Program (2018RQ24).

**ABBREVIATIONS**

IPTG isopropyl-beta-D-thiogalactopyranoside
ddH2O double-distilled water
q-RT PCR quantitative real-time PCR
RNR ribonucleotide reductase
RuSP ribose-5-phosphate
RSPr ribose-5-phosphate
PRPP phosphoribosyl pyrophosphate
IMP inosine 5’-monophosphate
XMP xanthosine 5’-monophosphate
GMP guanosine 5’-monophosphate
GDP guanosine 5’-diphosphate
dGDP 2’-deoxyguanosine 5’-diphosphate
GTP guanosine 5’-triphosphate
AMP adenosine 5’-monophosphate

REFERENCES

(1) Ledesma-Amaro, R.; Santos, M. A.; Jiménez, A.; Revuelta, J. L. Microbial Production of Vitamins. *Microbial Production of Food Ingredients Enzymes and Nutraceuticals*; Woodhead Publishing: England, 2013; p 571–594.

(2) Thakur, K.; Tomar, S. K.; De, S. Lactic acid bacteria as a cell factory for riboflavin production. *Microb. Biotechnol.* 2016, 9, 441–451.

(3) Revuelta, J. L.; Ledesma-Amaro, R.; Lozano-Martínez, P.; Díaz-Fernández, D.; Buey, R. M.; Jiménez, A. Bioproduction of riboflavin: a bright yellow history. *J. Ind. Microbiol. Biotechnol.* 2017, 44, 659–665.

(4) Lin, Z.; Xu, Z.; Li, Y.; Wang, Z.; Chen, T.; Zhao, X. Metabolic engineering of *Escherichia coli* for the production of riboflavin. *Microb. Cell Fact.* 2014, 13, 104.

(5) Dmytruk, K. V.; Yatsyshyn, V. Y.; Sybirna, N. O.; Fedorovych, D. V.; Sibiry, A. A. Metabolic engineering and classic selection of the yeast *Candida famata* (*Candida fareri*) for construction of strains with enhanced riboflavin production. *Metab. Eng.* 2011, 13, 82–88.

(6) Schwechheimer, S. K.; Park, E. Y.; Revuelta, J. L.; Becker, J.; Wittmann, C. Biotechnology of riboflavin. *Appl. Microbiol. Biotechnol.* 2016, 100, 2107–2119.

(7) Duan, Y. X.; Chen, T.; Chen, X.; Zhao, X. M. Over-expression of glucose dehydrogenase improves cell growth and riboflavin production in *Bacillus subtilis*. *Biotechnol. Lett.* 2010, 85, 1907–1914.

(8) Wang, Z.; Chen, T.; Ma, X.; Shen, Z.; Zhao, X. Enhancement of riboflavin production with *Bacillus subtilis* by expression and site-directed mutagenesis of zwf and gnd gene from *Corynebacterium glutamicum*. *Bioresour. Technol.* 2011, 102, 3934–3940.

(9) Liu, S.; Kang, P.; Cui, Z.; Wang, Z.; Chen, T. Increased riboflavin production by knock-out of 6-phosphofructokinase I and blocking the Entner-Doudoroff pathway in *Escherichia coli*. *Biotecnol. Lett.* 2016, 38, 1307–1314.

(10) Wang, G.; Shi, T.; Chen, T.; Wang, X.; Wang, Y.; Liu, D.; Guo, J.; Fu, J.; Feng, L.; Wang, Z.; Zhao, X. Integrated whole-genome and transcriptome sequence analysis reveals the genetic characteristics of a riboflavin-overproducing *Bacillus subtilis*. *Metab. Eng.* 2018, 48, 138–149.

(11) Silva, R.; Aguiar, T. Q.; Domingues, L. Blockage of the pyrimidine biosynthetic pathway affects riboflavin production in *Asbya gossypii*. *J. Biotechnol.* 2015, 193, 37–40.

(12) Shi, T.; Wang, Y.; Wang, Z.; Wang, G.; Liu, D.; Fu, J.; Chen, T.; Zhao, X. Deregulation of purine pathway in *Bacillus subtilis* and its use in riboflavin biosynthesis. *Microb. Cell Fact.* 2014, 13, 101.

(13) Wang, X.; Wang, G.; Li, X.; Fu, J.; Chen, T.; Wang, Z.; Zhao, X. Directed evolution of adenylsuccinatase synthetase from *Bacillus subtilis* and its application in metabolic engineering. *J. Biotechnol.* 2016, 231, 115–121.

(14) Boumezbeu, A.-H.; Bruer, M.; Stoecklin, G.; Mac, M. Rational engineering of transcriptional riboswitches leads to enhanced metabolite levels in *Bacillus subtilis*. *Metab. Eng.* 2020, 61, 58–68.

(15) Shi, S.; Shen, Z.; Chen, X.; Chen, T.; Zhao, X. Increased production of riboflavin by metabolic engineering of the purine pathway in *Bacillus subtilis*. *Biochem. Eng. J.* 2009, 46, 28–33.

(16) el Kouni, M. H. Potential chemotherapeutic targets in the purine metabolism of parasites. *Pharmacol. Ther.* 2003, 99, 283–309.

(17) Saxild, H. H.; Nygaard, P. Genetic and physiological characterization of *Bacillus subtilis* mutants resistant to purine analogs. *J. Bacteriol.* 1987, 169, 2977–2983.

(18) Endo, T.; Uratani, B.; Freese, E. Purine salvage pathways of *Bacillus subtilis and effect of guanine on growth of GMP reductase mutants. J. Bacteriol.* 1983, 155, 169–179.

(19) Yamamoto, A.; Hirose, Y.; Shiro, T. Studies on the production of nucleosides by microorganisms. *J. Gen. Appl. Microbiol.* 1966, 12, 299–309.

(20) Terakawa, A.; Natsume, A.; Okada, A.; Nishihata, S.; Kuse, J.; Tanaka, K.; Takenaka, S.; Ishikawa, S.; Yoshida, K. I. *Bacillus subtilis* 5’-nucleotidases with various functions and substrate specificities. *BMC Microbiol.* 2016, 16, 249.

(21) Schuch, R.; Saxild, H. H.; Garibian, A.; Pigott, P. J.; Nygaard, P. Nucleosides as a carbon source in *Bacillus subtilis*; characterization of the drm-pupG operon. *Microbiology* 1999, 145, 2957–2966.

(22) Li, H.; Zhang, G.; Deng, A.; Chen, N.; Wen, T. De novo engineering and metabolic flux analysis of inosine biosynthesis in *Bacillus subtilis*. *Biotechnol. Lett.* 2011, 33, 1575–1580.

(23) Nygaard, P.; Duckert, P.; Saxild, H. H. Role of adenine deaminase in purine salvage and nitrogen metabolism and characterization of the ade gene in *Bacillus subtilis*. *J. Bacteriol.* 1996, 178, 846–853.

(24) Zhang, Y.; Stubbe, J. *Bacillus subtilis* class Iβ ribonucleotide reductase is a dimanganese (III)-tyrosyl radical enzyme. *Biochemistry* 2011, 50, 5615–5623.

(25) Saxild, H. H.; Brunstedt, K.; Nielsen, K. I.; Jarmer, H.; Nygaard, P. Definition of the *Bacillus subtilis* PurR operon using genetic and bioinformatic tools and expansion of the PurR regulon with glyA, guaC, pbgG, xpt-pbuX, yqfZ-foiD, and pbuO. *J. Bacteriol.* 2001, 183, 6175–6183.

(26) Li, E. H. Whole Genome Sequencing and Analysis of Two Purine Nucleoside Industrial Producers. Master Thesis, Jiangxi Normal University, China, June 2015.

(27) Huangthong, M.; Herbig, A. F.; Bsat, N.; Helmann, J. D. Regulation of the *Bacillus subtilis* fur and perR genes by PerR: not all members of the PerR regulon are peroxide inducible. *J. Bacteriol.* 2002, 184, 3276–3286.

(28) Castro-Cerritos, K. V.; Yasin, R. E.; Robleto, E. A.; Pedraza-Reyes, M. Role of Ribonucleotide Reductase in *Bacillus subtilis* Stress-Associated Mutagenesis. *J. Bacteriol.* 2017, 199, 715–716 DOI: 10.1128/JB.00715-16.

(29) Liu, S.; Endo, K.; Ara, K.; Ozaki, K.; Ogasawara, N. Introduction of marker-free deletions in *Bacillus subtilis* using the AraR repressor and the ara promoter. *Microbiology* 2008, 154, 2562–2570.

(30) Livak, K. J.; Schmittgen, T. D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods* 2001, 25, 402.