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# Transcriptome analysis guided metabolic engineering of *Bacillus subtilis* for riboflavin production

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ABSTRACT

A comparative transcriptome profiling between a riboflavin-producing *Bacillus subtilis* strain RH33 and the wild-type strain *B. subtilis* 168 was performed, complemented with metabolite pool and nucleotide sequence analysis, to rationally identify new targets for improving riboflavin production. The *pur* operon (*purEKBCSQLFMNHD*) together with other PurR-regulated genes (*glyA*, *guaC*, *pbuG*, *xpt-pbuX*, *yqhZ-folD*, and *pbuO*) was all down-regulated in RH33, which consequently limited the supply of the riboflavin precursors. As 5-phospho-ribosyl-1(*a*)-pyrophosphate (PRPP) strongly inhibits the binding of PurR to its targets, it was inferred that the reduced expression of PurR-regulated genes might be caused by a low PRPP pool, which was subsequently confirmed by metabolite analysis. Thus, we selected and co-overexpressed *prs* and *ywlF* genes in RH33, which are involved in the biosynthetic pathway of PRPP from ribulose-5-phosphate. This co-amplification led to an elevated PRPP pool and thus the increased transcript abundances of PurR-regulated genes participated in riboflavin precursor biosynthesis. The riboflavin titer was increased by 25% (up to 15 $g\,l^{-1}$) in fed-batch fermentation.



## 1. Introduction

In the biotechnology industry, classical strain improvement has achieved a long history of success (Crueger and Crueger, 1984; Peberdy, 1985), which relied on random mutagenesis and selection. These methods are still very useful, especially with the development of efficient strain selection methods (Gall et al., 2008). However, unwanted changes in physiology and growth retardation may occur alongside the desired improvements. Since the introduction of metabolic engineering, a more rational improvement approach emerges for microbial development (Bailey, 1991; Stephanopoulos et al., 1998).

Riboflavin (vitamin B2) serves as a precursor for the synthesis of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are used as electron acceptors for many oxidoreductases (Stahmann et al., 2000). As such, riboflavin is required for a wide variety of cellular processes and is supplemented for feed and food fortification purposes in humans and animals to maintain health. The Gram-positive bacterium *Bacillus subtilis*, currently the most competitive riboflavin producer, has been widely adopted in the commercial riboflavin production processes (Knorr et al., 2007; Wu et al., 2007; Zamboni et al., 2003). The biosynthesis of riboflavin in *B. subtilis* occurs through seven enzymatic steps starting from GTP and ribulose-5-phosphate, which is shown in Fig. 1. The riboflavin producer *B. subtilis* was initially developed using the "classical" strain development approach that relied on iterative cycles of random mutagenesis and selection to create genetic diversity and identify improved riboflavin mutants (Perkins et al., 1991; Stahmann et al., 2000). Then, a number of conceivable strategies were carried out to construct high-level riboflavin-producing *B. subtilis* strains. These include enhancement of both gene dosages and transcriptional level of riboflavin operon in the mutants (Perkins et al., 1999), constitutive expression of the key gene (*ribA*) in riboflavin biosynthetic pathway (Hűmbelin et al., 1999), enhancing energy generation and reducing maintenance metabolism (Zamboni et al., 2003), increasing precursor supply by modulating carbon flow through pentose phosphate pathway (Zamboni et al., 2004a; Zhu et al., 2006), and deregulating *gapB* expression by *ccpN* knockout based on screening *B. subtilis* transposon mutants (Tännler et al., 2008).

However, these strategies were not based on a comprehensive analysis of the complex microbial metabolism and regulation, which would further facilitate the successful and efficient

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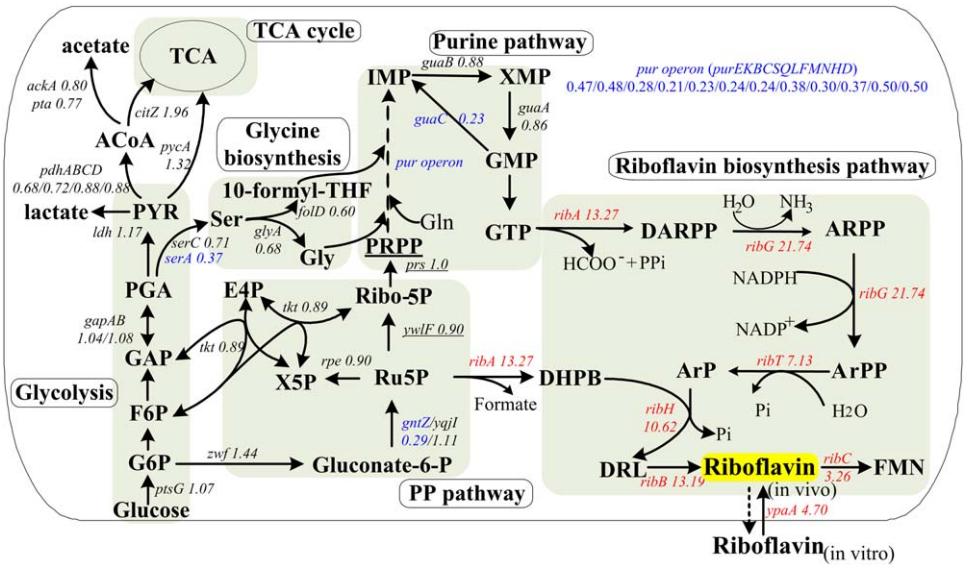


**Fig. 1.** Schematic overview of expression profiles of genes involved in relevant pathways of riboflavin production. The numbers are the ratios of the comparative expression levels in *B. subtilis* strain RH33 vs. 168. Blue lettering indicates downregulation, red indicates up-regulation, and black indicates without notable changes. The genes that were underlined were selected for overexpression. G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; GAP, D-glyceraldehyde 3-phosphate; PGA, 3-phosphoglycerate; PYR, pyruvic acid; AcoA, acetyl coenzyme A; Ser, serine; Gly, glycine; 10-formyl-THF, 10-formyl tetrahydrofolate; gluconate-6-P, 6-phospho-D-gluconate; Ru-5-P, ribulose-5-phosphate; Ribo-5-P, ribose-5-phosphate; PRPP, phosphoribosylpyrophosphate; Gln, glutamine; X5P, xylulose 5-phosphate; E4P, D-erythrose 4-phosphate; IMP, inosine monophosphate; XMP, xanthosine monophosphate; GMP, guanosine mono-phosphate; GTP, guanosine tri-phosphate; DARPP, 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone-5′-phosphate; ARPP, 5-amino-6-(5′-phosphoribosylamino)uracil; ArPP, 5-amino-6-(5′-phosphoribitylamino)uracil; ArP, 4-(1-D-ribitylamino)-5-amino-2,6-dihydroxypyrimidine; DHPB, 3,4-dihydroxy-2-butanone 4-phosphate; DRL, 6,7-dimethyl-8-ribityl-lumazine; FAD, flavin adenine dinucleotide (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

microbial metabolic engineering (Nielsen and Olsson, 2002). Recently, as a holistic and discovery-driven approach, transcriptome analysis has been successfully applied in metabolic engineering for rationally probing the complex gene and metabolic regulatory networks. Novel target genes have been identified to optimize microbial production strains (Choi et al., 2003; Harris et al., 2009; Izallalen et al., 2008; Jaluria et al., 2007; Park et al., 2007; Peebles et al., 2009; Sindelar and Wendisch, 2007; Wierckx et al., 2008).

Here we took a strategy to increase riboflavin production in a riboflavin-producing *B. subtilis* strain based on comparative transcriptome analysis between a riboflavin high-producer RH33 and wild type 168, integrated with DNA sequencing and metabolite pool measurement. Our integrated approach allowed us to understand genome-wide transcriptional differences underlying strain performance, and to identify potential metabolic bottlenecks for riboflavin production. We rationally overexpressed two genes simultaneously to activate precursor purine biosynthesis pathways by modulating global regulator activity via metabolite pool manipulation. This strategy was capable of elevating riboflavin titer by 25% in *B. subtilis* RH33 (up to 15 g $l^{-1}$) in fed-batch fermentation.

## 2. Materials and methods

### 2.1. Strains and plasmids

Bacterial strains and plasmids used in this work are listed in Table 1. Two different riboflavin-producing mutants *B. subtilis* RH13 and *B. subtilis* J617 were derived from *B. subtilis* 168 by multiple rounds of selection with azaguanine ($Az^r$), decoyinine ($Dc^r$) and roseoflavin ($RoF^r$) for resistance mutations that deregulate the riboflavin biosynthetic pathway. An integration vector pRB63 was constructed by inserting a native *rib* operon into pDG364 (BGSC, http://www.bgsc.org/). Then the vector pRB63 was integrated into the *B. subtilis* RH13 chromosome at the native *rib* operon locus by single crossover and selected at 5 μg $ml^{-1}$ chloramphenicol. Subsequently, the copy number of the integrated pRB63 was increased by selecting colonies that grew at higher chloramphenicol concentrations. Colonies that were able to grow up with 40 μg $ml^{-1}$ chloramphenicol were isolated, and one was denoted as *B. subtilis* RH13::$[pRB63]_n$. Another

**Table 1**
Strains and plasmids used in this study.

| Strain or plasmid | Description of genotype | Source |
|---|---|---|
| Strains | | |
| *B. subtilis* 168 | Wild-type | BGSC |
| *B. subtilis* RH33 | $Em^r$, $Cm^r$, containing multiple riboflavin operons | Laboratory stock |
| *B. subtilis* RH33-Prs | $Em^r$, $Cm^r$, $Spc^r$, containing a P43-*prs* (CDS) fragment integrated in the chromosome of RH33 | This study |
| *B. subtilis* RH33-PY | $Em^r$, $Cm^r$, $Spc^r$, containing a P43-*prs-ywlF* (CDS) fragment integrated in the chromosome of RH33 | This study |
| *E. coli* Top10 | Host strain for constructing plasmids | Laboratory stock |
| Plasmids | | |
| pUC18 | $Amp^r$ | BGSC |
| pSG1192 | $Amp^r$, $Spc^r$ | BGSC |
| pHPL10 | pHP13, containing a P43 promoter | Laboratory stock |
| pRPU10 | $Amp^r$, containing a *prs* (CDS) fragment | This study |
| pRPU12 | $Amp^r$, containing a P43-*prs* (CDS) fragment | This study |
| pRPU13 | $Amp^r$, $Spc^r$, containing a P43-*prs* (CDS) fragment | This study |
| pRPU14 | $Amp^r$, containing a P43-*prs-ywlF* (CDS) fragment | This study |
| pRPU15 | $Amp^r$, $Spc^r$, containing a P43-*prs-ywlF* (CDS) fragment | This study |

BGSC, *Bacillus* Genetic Stock Center (http://www.bgsc.org/).