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Directed evolution of adenylosuccinate synthetase from *Bacillus subtilis* and its application in metabolic engineering

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ABSTRACT

Adenylosuccinate synthetase (EC. 6.3.4.4) encoded by *purA* in *Bacillus subtilis*, catalyzing the first step of the conversion of IMP to AMP, plays an important role in flux distribution in the purine biosynthetic pathway. In this study, we described the use of site saturation mutagenesis to obtain a desired enzyme activity of adenylosuccinate synthetase and its application in flux regulation. Based on sequence alignment and structural modeling, a library of enzyme variants was created by a semi-rational evolution strategy in position Thr238 and Pro242. Other than *purA* deletion, the leaky mutation $purA^{P242N}$ partially reduced the flux towards AMP derived from IMP and increased the riboflavin synthesis precursor GTP, while also kept the requirement of ATP synthesis for cell growth. $PurA^{P242N}$ was introduced into an inosine-producing strain and resulted in an approximately 4.66-fold increase in inosine production, from 0.088 ± 0.009 g/L to 0.41 ± 0.051 g/L, in minimal medium without hypoxanthine accumulation. These results underline that the directed evolution of adenylosuccinate synthetase could tailor its activities and adjust metabolic flux. This mutation may provide a promising application in purine-based product accumulation, like inosine, guanosine and folate which are directly stemming from purine pathway in *B. subtilis*.

1. Introduction

The *de novo* synthesis of purine nucleosides has been elucidated in *Bacillus subtilis* – starting from GTP (guanosine 5′-tri-phosphate) and Ru-5-P (ribulose-5-phosphate) and proceeding through pyrimidine and pteridine intermediates (Chatwell et al., 2006) (Fig. 1). The purine *de novo* pathway leads to the conversion of PRPP (5-phospho-α-D-ribosyl-1-pyrophosphate) and glutamine into IMP (inosine 5′-mono-phosphate), after which IMP can be transformed into AMP (adenosine 5′-mono-phosphate) or GMP (guanosine 5′-mono-phosphate). The pool of intracellular purine nucleotides is maintained under strict control, hence the purine *de novo* pathway is tightly regulated by transcription repression and inhibition mechanism. For transcriptional repression, extracellular purines negatively regulate the transcription of most genes encoding enzymes required for *de novo* ATP (adenosine 5′-tri-phosphate) and GTP synthesis. For feedback inhibition regulation, enzymatic activities such as PRPP aminotransferase, adenylosuccinate synthetase and IMP dehydrogenase are subjected to end product-feedback regulation (Jiménez et al., 2005; Smith et al., 1994; Zakataeva et al., 2012). Adenylosuccinate synthetase (EC. 6.3.4.4), encoded by *purA* in *B. subtilis*, catalyzes the first step of the conversion of IMP to AMP. Studies on guanosine- and inosine-producing *B. subtilis* showed a significant increase in productivity when *purA* gene was inactivated in complex medium (Asahara et al., 2010; Li et al., 2011; Qian et al., 2006). In addition, examples of riboflavin-producing *B. subtilis* mutants bred by conventional chemical mutagenesis demonstrated that inactivated adenylosuccinate synthetase could increase GTP biosynthesis (Abbas and Sibirny, 2011; Shi et al., 2014). It is not surprising that regulation of *purA* expression should contribute to controlling *de novo* AMP synthesis and to balance the intracellular concentration of AMP and GMP.

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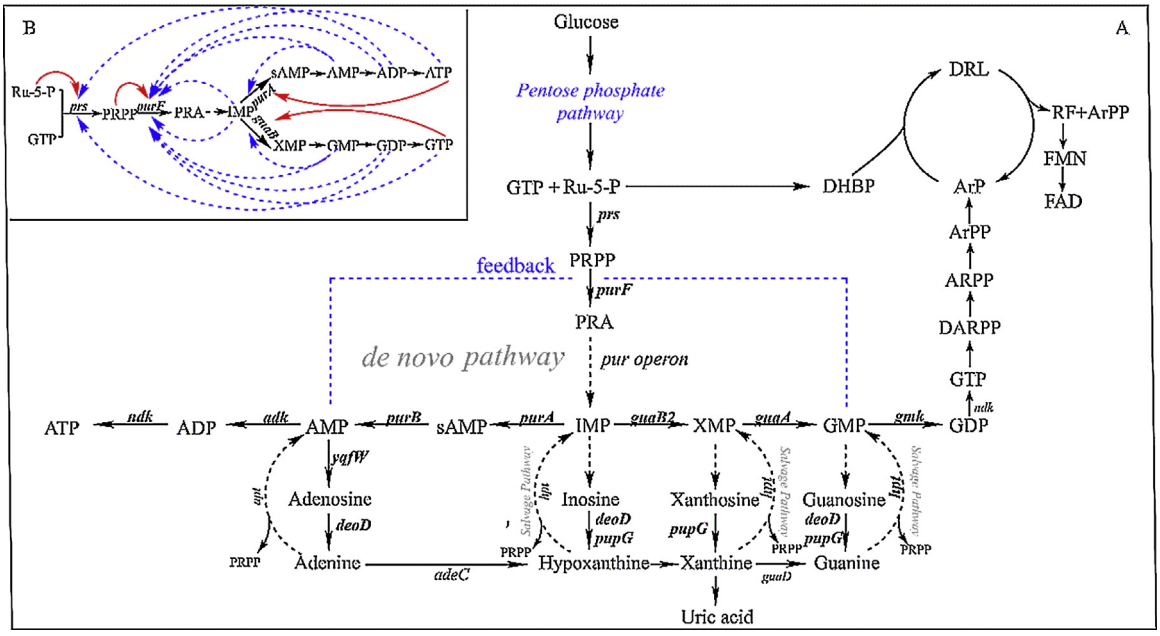

Fig. 1. Metabolic map of the purine biosynthetic pathway and the relationship of regulation. **(A)** Schematic overview of *de novo* purine biosynthetic pathway in *B. subtilis*. **(B)** Feedback inhibition regulation of purine biosynthetic pathway at metabolic levels. The red solid lines represent feedback inhibition, the blue dotted lines depict enzyme activation. Abbreviations: Ru-5-P, Ribose-5-phosphate; PRPP, 5-phospho-α-D-ribosyl-1-pyrophosphate; PRA, 5-phospho-α-D-ribosylamin; IMP, inosine 5′-mono-phosphate; XMP, xanthosine 5′-mono-phosphate; GMP, guanosine 5′-mono-phosphate; GDP, guanosine 5′-di-phosphate; GTP, guanosine 5′-tri-phosphate; sAMP, succinyladenosine mono-phosphate; AMP, adenosine 5′-mono-phosphate; ADP, adenosine 5′-di-phosphate; ATP, adenosine 5′-tri-phosphate; DARPP, 2,5-diamino-6-amino-ribose-4(3H)-pyrimidinone-5-phosphate; ARPP, 5-amino-6-amino-ribose-2,4(1H,3H)-pyrimidinedione-5-phosphate; ArPP, 5-amino-6-amino-ribitol-2,4(1H,3H)-pyrimidinedione-5-phosphate; ArP, 5-amino-6-amino-ribitol-2,4(1H,3H)-pyrimidinedione; DHBP, L-3,4-dihydroxy-2-butanone-4-phosphate; DRL, 6,7-dimethyl-8-tetrahydro-2,4-dioxo-ribose alcohol pteridine; FMN, Riboflavin-5-phosphate; FAD, Flavin adenine dinucleotide; RF, riboflavin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

However, *purA* is an essential gene for cell growth, decreased activity of *purA* drastically decreases the growth rate, and a *purA* deletion strain could not grow in minimum medium without adding the expensive precursor adenine. Supplementing adenine cannot completely restore cellular growth, indicating a partial compensatory effect by the *salvage pathway* (Peifer et al., 2012). Hence, strains deficient in *purA* usually extend fermentation periods and are adverse to industrial application. Here, we report a semi-rational evolution of adenylosuccinate synthetase using site saturation mutagenesis (SSM) to balance the AMP/GMP flux, along with identification of mutant characteristics. Moreover, the potential metabolic engineering application of a leaky *purA* mutation was also investigated *via* an inosine producing *B. subtilis* strain.

2. Materials and methods

2.1. Strains, plasmids and media

The strains and plasmids used in this study were summarized in Table 1. The primers were shown in Supplementary Table 1. All *B. subtilis* strains were derived from the wild type *B. subtilis* 168. *Escherichia coli* DH5α was used as host strain for cloning and plasmid construction. The vector pSS was used as backbone for plasmid construction (Shi et al., 2014). When required, antibiotics were added to media at the following concentrations: 100 μg/mL ampicillin for *Escherichia coli* selection; 5 μg/mL chloramphenicol, 5 μg/mL neomycin and 5 μg/mL erythromycin for *B. subtilis* selection. 5-fluorouracil (5FU) was purchased from Sigma-Aldrich Corporation (Sigma-Aldrich, St Louis, MO, USA) and prepared as a stock solution of 100 mM in dimethylsulfoxide (DMSO). MM medium (Wang et al., 2011) and M9 medium (Harwood and Cutting, 1990) were used for physiological characterization of riboflavin-producing and inosine-producing strains, respectively. Colonies that popped out the *upp*-cassette were selected on MM plate containing 10 μM 5FU.

2.2. Fermentation conditions and product analysis

In brief, to test the riboflavin biosynthetic activity of strains, single colony was transferred into 5 ml LB medium containing corresponding antibiotics and incubated at 37 °C in a rotatory shaker at 220 rpm for 10 h to prepare the inocula. The inocula was then added aseptically to flask (500 ml) containing 50 ml M9 medium (initial OD_{600} = 0.05). The fermentation was incubated at 41 °C in shake flasks at 240 rpm for 24 h. Cell growth was monitored by measuring optical density (OD) at 600 nm (OD_{600}). Glucose concentration in culture broth was determined enzymatically by a bioanalyzer (SBA-40E, Shandong, China). All the experiments were carried out independently in biological triplicates, and the reported results are the average of three replicate experiments. Measurement of riboflavin was described by Wang et al. (2011). Inosine and hypoxanthine concentrations in the culture broth were measured by reverse phase high-pressure liquid chromatography (RP-HPLC, HP 1100, Agilent Technologies, USA) at room temperature. The mobile phase was 4% (v/v) acetonitrile at a rate of 0.8 ml/min. The UV wavelength was 260 nm for inosine and hypoxanthine.

2.3. Strains and plasmids construction

The isolation and manipulation of recombinant DNA were performed using standard protocols (Sambrook and Russell, 2001). *B. subtilis* transformation and strain construction were performed according to a mutation delivery system as described previously by Shi et al. (2013). $RibC^{G199D}$ point mutation was introduced into *B subtilis* 168 to accumulate riboflavin, which was denoted as BS13 and used as host strain for screening *purA* mutations. Subsequently,