

# De novo engineering and metabolic flux analysis of inosine biosynthesis in *Bacillus subtilis*

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**Abstract** Wild-type *B. subtilis* strain W168 was de novo engineered for inosine biosynthesis. Inactivation of *deoD* and *purA* led to  $0.15 \pm 0.04$  and  $6.44 \pm 0.39$  g inosine/l yields, respectively. The *deoD purA* double mutant accumulated  $7.6 \pm 0.34$  g inosine/l, with a 4.7% (w/w) conversion ratio from glucose to inosine. Comparative metabolic flux analysis revealed that the fluxes from inosine to hypoxanthine and from inosine monophosphate to adenosine monophosphate in the double mutant decreased to 14.0 and 0.61% of those in the wild-type strain. The major role of *purA* was demonstrated when inactivation of *deoD* and *purA* were found to contribute additively to inosine accumulation. This work is expected to contribute to

the improvement of the fermentative production of purine nucleosides in the microbial industry.

**Keywords** *Bacillus subtilis* · Fermentation · Genetic engineering · Inosine

## Introduction

Inosine is a commercially important flavor enhancer in the food industry and is a precursor for anti-viral drug synthesis in the pharmaceutical industry (Torii et al. 2010). It has been produced in bulk by *B. subtilis* for decades. However, the majority of industrial strains were screened by conventional mutagenesis approaches, which would have introduced undesirable secondary mutations. Recently, the wild-type *Escherichia coli* strain W3110 has been subjected to rational engineering for inosine production. Inactivation of *edd*, *pgi*, *xpa*, and *guaA* (Shimaoka et al. 2005; Shimaoka et al. 2006) and overexpression of desensitized *purF* and *prs* (Shimaoka et al. 2007) have been reported to enhance inosine accumulation in *E. coli*.

With the publication of the initial and re-sequenced genome of the laboratory workhorse *B. subtilis* 168 (Kunst et al. 1997; Srivatsan et al. 2008) and its amenability for genetic manipulation, *B. subtilis* has become attractive for engineering to produce various metabolites (Wu et al. 2009).

Here, we report the de novo engineering of *B. subtilis* W168 for the overproduction of inosine.

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By sequential inactivation of genes encoding branch point enzymes in the inosine shunt pathway, a series of inosine overproducing strains were constructed. The effect of *deoD* and *purA* inactivation on inosine accumulation was also evaluated by metabolic flux analysis.

## Materials and methods

### Inactivation of *deoD* and *purA* in *B. subtilis* W168

The strains and plasmids used in this study are listed in Supplementary Table 1. Inactivation of *deoD* and *purA* were performed by double crossover homologous recombination. The *deoD* knockout plasmid was constructed by inserting the upstream and downstream regions of *deoD*, as well as the kanamycin resistance cassette from pDG780, into pBluescript II SK (-) in a sequential manner, to yield pWYE753. The *purA* knockout plasmid was constructed by inserting the upstream and downstream region of *purA* into pDG641 to generate pWYE761. The PCR primers, including restriction sites, are listed in Supplementary Table 2.

Transformation of *B. subtilis* was carried out using the natural genetic competence method as described previously (Anagnostopoulos and Spizizen 1961). The *deoD* was inactivated by transformation of strain W168 with pWYE753 and selection on solidified Luria–Bertani (LB) containing 10 µg kanamycin/ml. The *deoD* knockout mutant was termed BS017. *purA* was knocked out by transformation of strain W168 with pWYE761 and selecting on 0.5 µg erythromycin/ml plus 12.5 µg lincomycin/ml (MLS). The *purA*-disrupted mutant was termed BS018. The *deoD* and *purA* double mutant, BS019, was generated by transforming strain BS017 with the chromosomal DNA of BS018, and selecting for kanamycin and MLS resistance. All of the mutants were verified by PCR and sequencing.

### Shake-flask fermentation

*B. subtilis* mutants were cultured in seed medium at 32°C and 200 rpm until the OD<sub>600</sub> reached 12. The seed culture was then inoculated into 30 ml of fermentation medium in a 500 ml shake-flask at a ratio of 10% (v/v). Fermentation was carried out at

36°C and 200 rpm for 75 h. The pH was controlled at 6.4–7.0 by supplementation with ammonia.

### Analytical methods

Glucose and lactic acid were determined by an SBA-40D biosensor analyzer (Institute of Biology of Shandong Province Academy of Sciences, Shandong, China). Inosine, guanosine monophosphate, and hypoxanthine were measured using HPLC equipped with a Zorbax SB-Aq column (4.6 × 250 mm, 5 µm; Agilent) at 33°C. 0.5% KH<sub>2</sub>PO<sub>4</sub> (pH 4.5) was used for elution, and the flow rate was controlled at 1 ml min<sup>-1</sup>. Purine compounds were detected at 254 nm. L-alanine and L-aspartic acid were also measured by HPLC using a pre-column derivatization method, as described previously (Chen et al. 2009).

### Metabolic flux analysis (MFA)

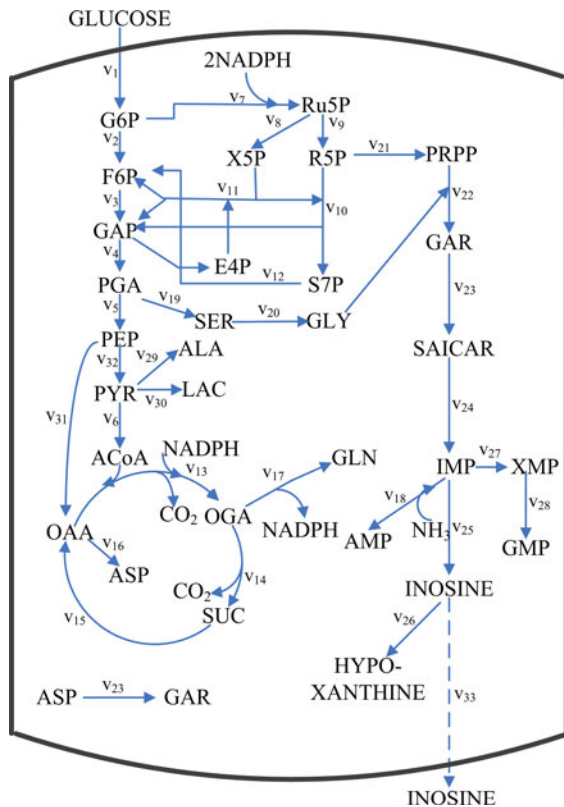
The stoichiometric model of intracellular metabolism was constructed according to previous reports, with slight modifications (Sauer et al. 1998), and is shown in Fig. 1. The stoichiometric functions and the metabolites involved in the reactions are listed in Supplemental Tables 3 and 4, respectively.

MFA of *B. subtilis* was based on three assumptions. Firstly, the cells would cease growth during the last period of fermentation, which means that the biomass would not increase or decrease, and this period was named the Pseudo-steady state (PSS) (Bai et al. 2004). Secondly, NADPH and NADP<sup>+</sup> are equivalent. Lastly, the consumption energy is not equivalent to maintenance energy, owing to the production of futile energy and metabolism exhibiting no clear function; therefore, the ATP energy equivalent was not considered. The total metabolic fluxes can be depicted by the following equation (Eq. 1):

$$A \cdot v_j - \mu \cdot x_i = \frac{dc_i(t)}{dt} \quad (1)$$

$$(i = 1, 2, 3, \dots, m; j = 1, 2, 3, \dots, n)$$

where,  $A$  represents the  $m \times n$  stoichiometric matrix of intracellular metabolism, in which the row specifies each compound and the column specifies each reaction in Fig. 1;  $\mu$  is the change of biomass, and  $x_i$  is the intracellular concentration of compound  $i$ ;



**Fig. 1** Metabolic network for *B. subtilis* inosine biosynthesis. *G6P*, glucose 6-phosphate; *F6P*, fructose 6-phosphate; *GAP*, glyceraldehyde 3-phosphate; *PGA*, 3-phosphoglyceric acid; *PEP*, phosphoenolpyruvic acid; *PYR*, pyruvic acid; *ACoA*, acetyl coenzyme A; *OAA*, oxaloacetic acid; *SUC*, succinic acid; *ASP*, L-aspartic acid; *OGA*, oxoglutaric acid; *GLN*, L-glutamine; *ALA*, L-alanine; *LAC*, lactic acid; *SER*, L-serine; *GLY*, glycine; *E4P*, erythrose 4-phosphate; *S7P*, sedoheptulose 7-phosphate; *Ru5P*, ribulose 5-phosphate; *X5P*, xylulose 5-phosphate; *R5P*, ribose 5-phosphate; *PRPP*, phosphoribosyl pyrophosphate; *GAR*, glycinamide ribonucleotide; *SAICAR*, phosphoribosylaminoimidazolesuccinocarboxamide; *IMP*, inosine monophosphate; *XMP*, xanthosine monophosphate; *GMP*, guanosine monophosphate; *AMP*, adenosine monophosphate

$c_i$  (mM) is the extracellular concentration of compound  $i$ .

According to the PSS assumption,  $\mu$  is zero in the last period of fed-batch fermentation (64–72 h). Eq. 1 can be reduced to

$$A \cdot v_j = \frac{dc_i(t)}{dt} = \sum_{j=1}^n a_{ij} \cdot v_j \quad (2)$$

$$(i = 1, 2, 3, \dots, m; j = 1, 2, 3, \dots, n)$$

where  $a_{ij}$  is the coefficient of compound  $i$  in reaction  $j$ .

The stoichiometric model in this study (Supplementary Tables 3 and 4) consists of 33 reactions and 26 compounds; the degree of freedom (DF) is  $33 - 26 = 7$ , which means that the changing rate (mM/h) of seven other compounds should be measured to determine all of the reaction rates in the metabolic network ( $v_j$ ,  $j = 1, 2, 3, \dots, 34$ ). Glucose, inosine, hypoxanthine, GMP, AIA, ASP, and lactic acid were chosen for quantification. All of the reaction rates (mM/h) were normalized to the glucose uptake rate, which was defined as 100%. The matrix function was solved using MATLAB 7.0 (MathWorks).

## Results

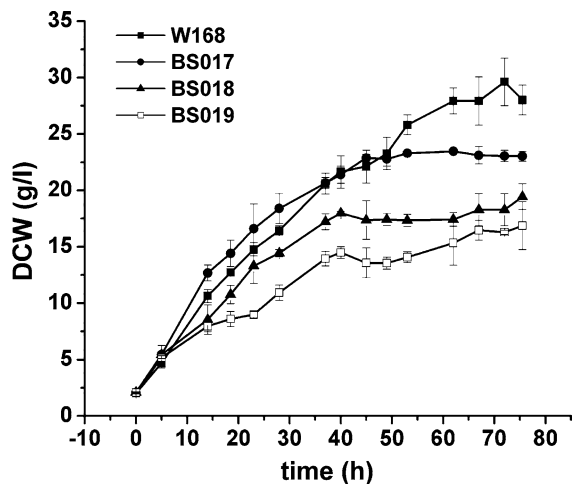
### Cell growth, glucose consumption, and inosine accumulation

Inactivation of *purA* exhibited a dramatic retarding effect on biomass (Fig. 2) and glucose consumption rate (Fig. 3). Both BS018 and BS019 showed slower growth and lower glucose consumption rates than those of W168 and BS017. Inactivation of *purA* also exhibited the most potent effect on inosine accumulation (Fig. 4). Wild-type W168 produced  $0.044 \pm 0.019$  g inosine/l; strain BS017 and BS018 accumulated  $0.15 \pm 0.04$  and  $6.44 \pm 0.39$  g inosine/l, respectively. The double mutant BS019 accumulated  $7.6 \pm 0.34$  g inosine/l, representing a 4.7% (w/w) conversion ratio from glucose to inosine. The inosine yield of BS019 was improved 172.7-fold compared with the parental strain W168.

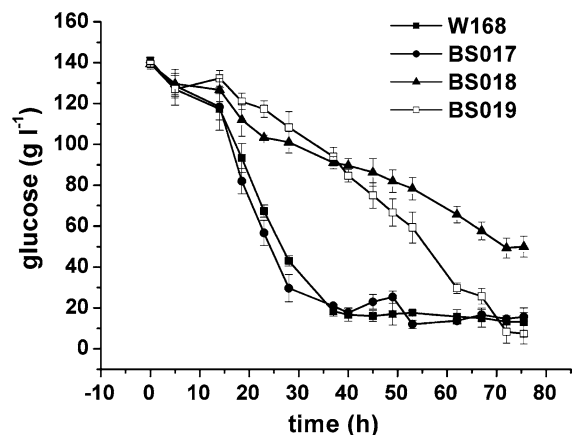
### Comparative MFA

The flux distributions of the major metabolic pathways in *B. subtilis* W168 and the inosine-producing mutants were compared, including the EMP, the HMP, the TCA cycle, the purine de novo synthesis pathway, and the partial purine salvage pathway.

The relative fluxes of each intracellular metabolic reaction are presented in Fig. 5, where they were normalized to glucose uptake rate (100%). Dramatic changes in the fluxes from inosine to hypoxanthine, and from IMP to AMP, were observed between the parental strain and the mutants, as indicated by  $v_{26}$  and  $v_{18}$ . The ratio of flux  $v_{26}$  in strain BS017 to that in W168 was 15.8% (0.09/0.57%), and the ratio of flux

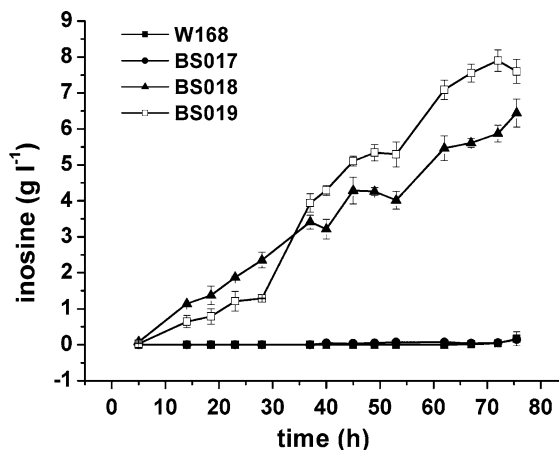


**Fig. 2** Cell growth curves during shake-flask fermentation of strain W168 and its mutants. Fed-batch fermentation was performed in 500 ml shake-flasks containing 30 ml of fermentation medium. The seed medium contained 20 g glucose/l, 10 g tryptone (Oxoid)/l, 15 g yeast powder/l, 7 g corn steep liquor/l, 2.5 g NaCl/l, and 2 g urea/l, pH 7.0–7.2. The fermentation medium contained 140 g glucose/l, 16 g corn steep liquor/l, 15 g yeast powder/l, 13 g urea/l, 22 g  $(\text{NH}_4)_2\text{SO}_4$ /l, 4 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ /l, 5 g  $\text{K}_2\text{HPO}_4$ /l, and 20 g  $\text{CaCO}_3$ /l, pH 7.0–7.2. Dry cell weight (DCW) was calculated on the base of  $\text{OD}_{600}$  ( $1 \text{ OD}_{600} = 0.35 \text{ g}_{\text{DCW}}/\text{l}$ ). Data shown are averages of at least three independent experiments, with standard deviations



**Fig. 3** Plots of glucose concentration against time during shake-flask fermentation. Glucose concentrations were determined using an SBA-40D biosensor analyzer. Residual glucose was controlled at 10–20% by supplementation from an 80% glucose stock solution. Data shown are averages of at least three independent experiments, with standard deviations

$v_{18}$  in strain BS018 to that in W168 was 13.3% (0.65/4.88%). Despite no dramatic difference in the reduction rate of corresponding metabolic flux, the



**Fig. 4** Plots of inosine yield against time during shake-flask fermentation of strain W168 and its mutants. Inosine was measured by the HPLC method. Inosine yields shown are averages of at least three independent experiments, with standard deviations

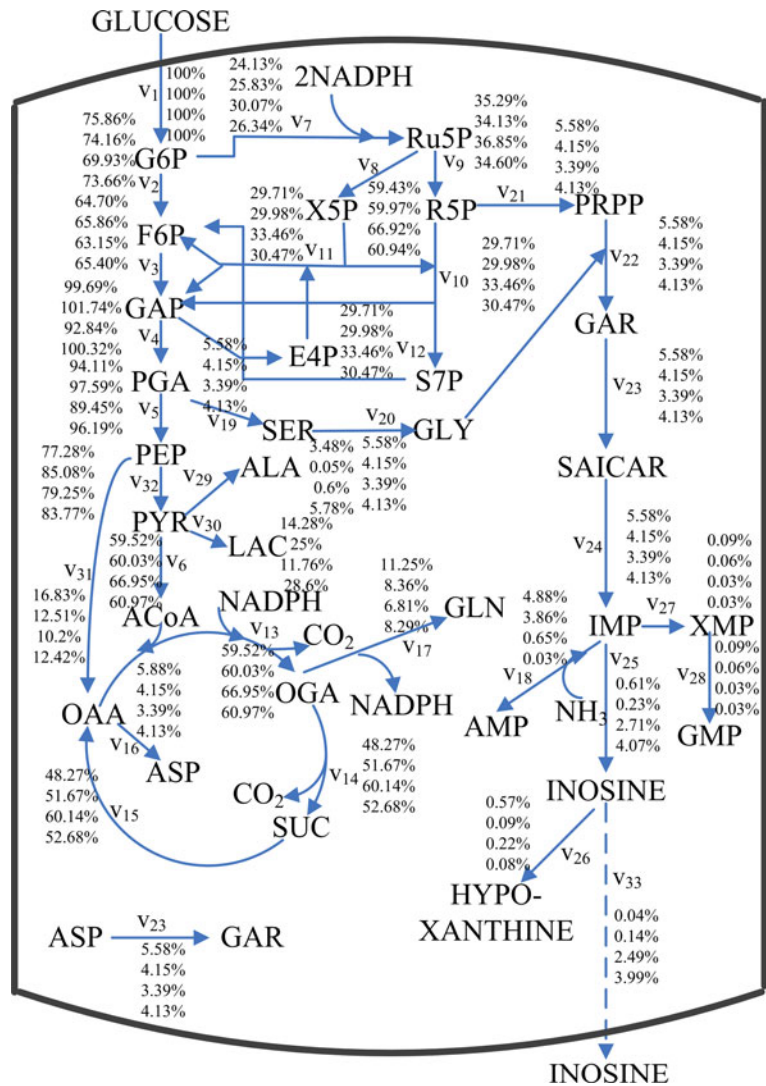
inosine yield of strain BS018 exceeded that of BS017 by 42.8-fold. Inactivation of *purA*, therefore, had a major effect on inosine accumulation. Moreover, the fluxes of  $v_{26}$  and  $v_{18}$  in strain BS019 were 14.0 and 0.03% compared to those of W168, with a further reduction of  $v_{18}$  flux in the double mutant strain. The increase of inosine yield in strain BS019 compared to that in BS018 suggests that inactivation of *purA* and *deoD* have an additive effect on inosine accumulation, and the effect of *deoD* inactivation could only be observed in a *purA* deficient background.

The fluxes of the TCA cycle ( $v_{13}$ ,  $v_{14}$ ,  $v_{15}$ ), EMP ( $v_2$ ), and HMP ( $v_7$ ) did not significantly respond to the inactivation of *deoD* or *purA*. This suggested that local flux re-distribution might not disturb the global flux network in cellular metabolism, i.e., various regulatory machineries could respond to maintain the global metabolic balance. Likewise, the synthesis fluxes of precursor amino acids used in de novo purine synthesis, such as glycine ( $v_{20}$ ) and aspartic acid ( $v_{16}$ ), did not increase. Therefore, these amino acids may not be the rate-limiting factors in inosine biosynthesis.

## Discussion

We have performed de novo genetic engineering of the laboratory workhorse *B. subtilis* W168 for inosine biosynthesis. The *deoD* and *purA* double mutant

**Fig. 5** Comparative analysis of flux distributions in strains W168, BS017, BS018, and BS019. The relative fluxes were normalized to the absolute glucose uptake rate of each mutant (15.6, 18.9, 7.8, and 17.8 mM/h for strains W168, BS017, BS018, and BS019, respectively), where glucose uptake is defined as 100%. For each reaction, the four percentages from top to bottom are for W168, BS017, BS018, and BS019, respectively



could produce 7.6 g inosine/l. Recently, 6.7 g inosine/l was accumulated after nine genes that potentially hindered inosine accumulation were knocked out and two desensitized genes participating in inosine synthesis were overexpressed in *E. coli* W3110 (Shimaoka et al. 2007). This dramatic difference may be caused by the intrinsic metabolic divergence between *E. coli* and *B. subtilis*. It has been reported that *Bacillus* exhibit stronger HMP flux than other bacterial genera; therefore, more PRPP, which is the major precursor for purine nucleoside and riboflavin synthesis, could be accumulated (Sauer et al. 1998). Thus, *B. subtilis* appears to be more appropriate for the production of nucleosides, riboflavin, and their derivatives (Duan et al. 2010).

MFA was efficient in evaluating the effects of genetic engineering. The decreased fluxes from inosine to hypoxanthine and from IMP to AMP together revealed the successful re-direction of purine compound local inter-conversion by genetic engineering. The steady fluxes of the TCA cycle, EMP, and HMP revealed the complexity in cellular metabolic regulation. It has been predicted that the optimal flux ratio of HMP to EMP should be 34.3/65.7% in a guanosine producing strain (Sauer et al. 1998). In the current study, this ratio was 26.34/73.66% in strain BS019, which is lower than the theoretical value. Thus, these major metabolic pathways appear to be appropriate targets for strain improvement by systems engineering in future research.

Inactivation of *deoD* did not contribute as much to inosine accumulation as *purA* in this study, which may be caused by the redundant activity of purine nucleoside phosphorylase, which is encoded by *pupG* (Schuch et al. 1999). Further inactivation of *pupG* may cause the complete loss of purine nucleoside phosphorylase activity in *B. subtilis*, which would increase inosine accumulation. In addition to *deoD* and *purA*, many other target genes in the purine synthesis pathway have been reported to increase inosine production when inactivated or overexpressed, including *purR*, *pgi*, *prs*, *purF* (Shimaoka et al. 2007), and the gene encoding the purine nucleoside efflux pump, *pbuE* (Zakataeva et al. 2007). However, introducing so many mutations into the same strain background appears labor intensive, and novel genetic manipulation techniques, such as multiplex automated genome engineering (MAGE), may accelerate this process. Wang et al. 2009 succeeded in modifying 24 target genes simultaneously using MAGE, in an effort to elevate lycopene production in *E. coli*. MAGE may also be applied for engineering *B. subtilis* for inosine production in the future.

## Conclusion

The *deoD* and *purA* double mutant *B. subtilis* strain (BS019) could accumulate  $7.6 \pm 0.34$  g inosine/l in shake-flask fermentation—productivity comparable to that obtained by strains generated by traditional mutagenesis in industry. Subsequent MFA revealed a successful re-directing of metabolic flux in purine compound inter-conversion pathways. Therefore, strain BS019 may serve as an appropriate candidate for further improvement of inosine biosynthesis by a systems metabolic engineering approach.

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