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# Inhibition of glycine cleavage system by pyridoxine 5′-phosphate causes synthetic lethality in *glyA yggS* and *serA yggS* in *Escherichia coli*

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## Abstract

The YggS/Ybl036c/PLPBP family includes conserved pyridoxal 5′-phosphate (PLP)-binding proteins that plays a critical role in the homeostasis of vitamin $B_6$ and amino acids. Disruption of members of this family causes pleiotropic effects in many organisms by unknown mechanisms. In *Escherichia coli*, conditional lethality of the *yggS* and *glyA* (encoding serine hydroxymethyltransferase) has been described, but the mechanism of lethality was not determined. Strains lacking *yggS* and *serA* (3-phosphoglycerate dehydrogenase) were conditionally lethality in the M9-glucose medium supplemented with Gly. Analyses of vitamin $B_6$ pools found the high-levels of pyridoxine 5′-phosphate (PNP) in the two *yggS* mutants. Growth defects of the double mutants could be eliminated by overexpressing PNP/PMP oxidase (PdxH) to decrease the PNP levels. Further, a *serA pdxH* strain, which accumulates PNP in the presence of *yggS*, exhibited similar phenotype to *serA yggS* mutant. Together these data suggested the inhibition of the glycine cleavage (GCV) system caused the synthetic lethality. Biochemical assays confirmed that PNP disrupts the GCV system by competing with PLP in GcvP protein. Our data are consistent with a model in which PNP-dependent inhibition of the GCV system causes the conditional lethality observed in the *glyA yggS* or *serA yggS* mutants.

## Graphical abstract

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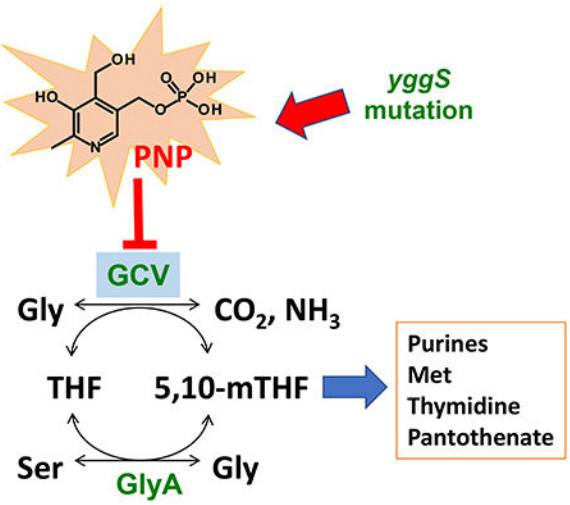


The YggS/Ybl036c/PLPBP family includes conserved pyridoxal 5′-phosphate (PLP)-binding proteins that plays a critical role in the homeostasis of vitamin $B_6$ and amino acids. In *Escherichia coli*, conditional lethality of the *yggS* and *glyA* (encoding serine hydroxymethyltransferase) has been described, but the mechanism of lethality was not determined. We demonstrated that PNP-dependent inhibition of the GCV system causes the conditional lethality observed in the *glyA yggS* mutants.

## Introduction

Pyridoxal 5′-phosphate (PLP) is the biologically active form of vitamin $B_6$, and is used as a cofactor in various kinds of enzymes in most organisms. PLP-dependent enzymes catalyze more than 140 distinct reaction types, including transamination, decarboxylation, racemization, and elimination or replacement of chemical groups at the $C_\beta$ or $C_\gamma$, and are involved in sugar, lipid, and amino acid metabolism (Percudani et al., 2003; Eliot et al., 2004; Toney, 2011). In addition to the coenzyme function, PLP may function as an antioxidant (Bilski et al., 2000) and modulator of steroid hormone (Tully et al., 1994) or P2X purinoceptor7 (Lambrecht et al., 2002; Thériault et al., 2014). Most of the bacteria utilize PLP-dependent transcriptional factors to modulate metabolisms of vitamin $B_6$ and/or amino acid (Belitsky, 2004; Belitsky, 2014; Takenaka et al., 2015; Tramonti et al., 2018).

Two different pathways, deoxyxylulose 5-phosphate (DXP)-dependent or DXP-independent pathways, are known for PLP biosynthesis (Laber et al., 1999; Fitzpatrick et al., 2007; di Salvo et al., 2011). The DXP-dependent pathway contains multiple reactions and is found in a few members of the γ-proteobacteria and *Escherichia coli*. This pathway utilizes 4-phospho-hydroxythreonine and DXP to synthesize pyridoxine 5′-phosphate (PNP) as a precursor of PLP. The DXP-independent pathway is found in archaea, fungi, most eubacteria, and plants, and PLP is formed directly from glutamine, ribose 5-phosphate/ribulose 5-phosphate, and glyceraldehyde 3-phosphate/dihydroxyacetone phosphate by the action of PLP synthase (Burns et al., 2005; Raschle et al., 2005; di Salvo et al., 2011; Fitzpatrick et al., 2007). Mammals do not possess the PLP biosynthetic pathway and thus obtain $B_6$ vitamers from their diet. In all organisms, $B_6$ vitamers are converted to PLP by a

salvage pathway that involves pyridox(am)ine 5′-phosphate oxidase (EC 1.4.3.5), phosphatases, and/or pyridoxal/pyridoxine kinase (EC 2.7.1.35) (Fitzpatrick et al., 2007; di Salvo et al., 2011). In mammals, PLP-dependent enzymes play essential roles in the metabolism of neurotransmitters including γ-aminobutyric acid, dopamine, epinephrine, serotonin, serine, and histamine. Therefore, a deficiency of PLP is implicated in neurological disorders. Excess PLP is thought to be toxic to mammals, probably due to the non-specific binding of reactive aldehyde group at C4′ to amines and/or thiols.

In man, disruption of $B_6$ homeostasis, including interfering with $B_6$ synthesis, recycling (PNPO deficiency), and transportation (tissue non-specific alkaline phosphatase deficiency), and inactivation of PLP by accumulation of a metabolite that reacts with PLP (ALDH7A1 or ALDH4A1 deficiency), causes vitamin $B_6$-dependent epilepsy, which is treatable with high-dose of $B_6$ vitamers such as pyridoxine (PN) and PLP (Farrant et al., 2001; Narisawa et al. 2001; Mills et al., 2005; Mills et al., 2006; Wilson et al., 2019). Darin et al. recently found that a mutation in PROSC, **pro**line **s**ynthetase **c**o-transcribed homolog protein (renamed as PLPBP) causes vitamin $B_6$-dependent epilepsy (Darin et al., 2016). The PLPBP-deficiency exhibits complex phenotypes including altered levels of vitamin $B_6$ vitamers and imbalances of neurotransmitters with evidence of reduced activity of PLP-dependent enzymes (Plecko et al., 2017; Shiraku et al., 2018; Johnstone et al., 2019).

PLPBP is a member of a highly conserved PLP-binding protein family (COG0325, referred to herein as YggS/Ybl036c/PLPBP) and the members of this protein family are present in three domains of life. The YggS/Ybl036c/PLPBP family exhibits typical TIM-barrel structure and the PLP is bound to a Lys residue through Schiff-base linkage (Eswaramoorthy et al., 2003; Ito et al., 2013; Tremiño et al., 2017). The PLP-binding is essential for the function of this protein family (Ito et al., 2013; Tremiño et al., 2018). In human cells, the PLPBP localizes in mitochondria and cytosol (Johnstone et al. 2019). Although this protein family shows structural similarity to bacterial alanine racemase and eukaryotic ornithine decarboxylase (Eswaramoorthy et al., 2003; Tremiño et al., 2017), representatives of fold-type III PLP-dependent enzyme, no enzymatic activity has been detected with the purified proteins (Ito et al., 2013). Previous studies showed that in *E. coli* mutation of this protein family (*yggS*) causes pleiotropic phenotypes, including altered flux in the Thr-Ile-Val metabolic pathways (Ito et al., 2013; Ito et al., 2016; Ito et al., 2019), increased sensitivity to PN, accumulation of pyridoxine 5′-phosphate (PNP) in the cells (Prunetti et al., 2016), and synthetic lethality with *glyA* (serine hydroxymethyltransferase) (Nichols et al., 2011; Côté et al., 2016; Prunetti et al., 2016). A recent study showed that the two phenotypes, PN sensitivity and the aberration of amino acid metabolism in Thr-Ile-Val pathway, are caused by high-levels of PNP via an unidentified mechanism (Ito et al., 2019). In cyanobacteria, disruption of the cyanobacterial member of this protein family (PipY) increases sensitivity to antibiotics targeting essential PLP-dependent enzymes and causes synthetic lethality with putative cysteine synthase CysK (Labella et al., 2017). Studies with the yeast strain lacking Ybl036c suggest a role of this protein in mitochondrial metabolism (Johnstone et al., 2019). The same authors demonstrated that the *plpbp*$^{-/-}$ zebrafish larvae exhibit seizure phenotype and impaired biosynthesis and/or homeostasis of PLP-dependent neurotransmitters (Johnstone et al., 2019).

Although the mutation of the YggS/Ybl036c/PLPBP family causes significant effects on the metabolisms of vitamin $B_6$ and amino acid, limited information is available for the molecular mechanisms. In this study, we undertook a detailed study about the conditional lethality of *glyA* and *yggS* observed in *E. coli.* GlyA catalyzes the PLP-dependent conversion of Ser to Gly while transferring the hydroxymethyl group to tetrahydrofolate (THF) to generate 5, 10-methylenetetrahydrofolate (5, 10-mTHF). The GlyA reaction provides most of Gly and 5, 10-mTHF in *E. coli* and the latter is required for Met, thymidine, and purine biosynthesis (Blaklet, 1955; Blakley 1969; Schirch et al., 1985) (Fig. 1). The reason why the *yggS* mutation induces lethality under the *glyA*-background remained to be elucidated.

Here we show that the *yggS* mutation under the *serA* (3-phosphoglycerate dehydrogenase)-background also causes conditional lethality in the M9-glucose medium supplemented with Gly. Further studies demonstrated that high-levels of PNP, which is induced by *yggS* mutation, disrupts glycine cleavage (GCV) system and causes lethality under the *glyA*- or *serA*-background. The PNP-dependent inhibition of the GCV system may provide a plausible reason for the diverse phenotypes observed in *E. coli*.

## Results and Discussion

### Poor growth of *glyA yggS* double mutant and its alleviation by inosine

Null alleles of *yggS* and *glyA* (encoding serine hydroxymethyl transferase; EC 2.1.2.1) in *E. coli* are synthetically lethal on LB medium, although both the single mutant grow well on the same medium (Nichols et al., 2011; Côté et al., 2016; Prunetti et al., 2016). We constructed a *glyA yggS* double mutant using LB medium supplemented with methionine and thymidine. These two compounds partially alleviate the growth defect of the double mutant on LB (Prunetti et al., 2016). In our hands, the double mutant grew in LB medium, although it showed severe growth defect (Fig. 2A). A previous study suggested that this LB sensitivity phenotype is due to amino acids such as Thr and Ala (Prunetti et al., 2016). The apparent inconsistency could be due to the different nutrients composition of the media that affect the growth of the *glyA yggS* mutant. Consistent with previous results, the *glyA yggS* mutant had a significant growth defect in an M9-glucose medium supplemented with 0.4% casamino acid (M9-Casa medium) when compared to the *glyA* mutant (Fig. 2B). Plasmid-borne expression of *yggS* completely restored the growth of the double mutant to the level of the *glyA* mutant, confirming the growth defect was due to the *yggS* mutation (Fig. 2B). The addition of Ser or Gly (5 mM) to the M9-Casa medium did not significantly affect the growth of the *glyA yggS* mutant (data not shown). These data suggested that neither Ser toxicity nor Gly limitation caused the growth defect in the *glyA yggS* double mutant.

In some conditions, a *yggS* mutant overproduces Val, which is toxic to *E. coli* K-12 (Ito et al., 2019). To ask if increased Val could be responsible for inhibition of growth of the *glyA yggS* mutant, the amino acid pool of the double mutant grown in the M9-Casa medium was analyzed. A significantly altered amino acid pool was found in the double mutant as compared to that of the *glyA* mutant strain (Fig. 2C). The *glyA yggS* double mutant accumulated Met, 2-AB, Val, Ile, and Leu compared to the *glyA* mutant (Fig. 2C). Ile alleviates Val toxicity in *E. coli* K-12 strain (Leavitt et al. 1962; Lawther et al., 1981), but

providing Ile (0.1 – 1 mM) to the M9-Casa medium did not improve the growth of the double mutant (data not shown). These results indicated that Val toxicity is not the cause of the growth defect in the *glyA yggS* mutant, which accumulated 2.5-times more Val than the *glyA* mutant.

Supplementation with a variety of metabolites found that guanosine or inosine (0.2 mM) eliminated the growth defect of the *glyA yggS* mutant in the M9-Casa medium (Fig. 3A, 3C). Adenosine was also effective for restoring the growth of the *glyA yggS* mutant (Fig. 3A). Supplementation of either of Gly, Met, thymidine, cytidine, or pantothenate (0.2 mM), or the combination of the two did not significantly support the growth of the double mutant under the condition examined (Fig. 3A, B, C). Inosine is probably phosphorylated to inosine-5′-phosphate (IMP) by a salvage pathway and used as a purine source in *E. coli* (Xi et al., 2000). The growth characteristics suggest that *yggS* mutation under the *glyA*-background influences the purine biosynthesis. Because the purine biosynthesis requires one-carbon unit metabolism, it was also possible that the one-carbon unit metabolism is affected in the *yggS* mutant.

## Growth of a *serA yggS* double mutant suggests compromised glycine cleavage (GCV) system

In a wild-type strain, GlyA and/or GCV system play role in the production of 5, 10-mTHF (Fig. 1), and a *glyA* mutation, in combination with a lesion in any of the genes of the GCV complex (*gcvT, gcvH, gcvP*, or *lpd*) is synthetically lethal (Côté et al., 2016). In the *glyA* mutant, the GCV system is responsible for the production of 5,10-mTHF (Kikuchi et al., 2008) (Fig. 1). The possibility that the GCV system was inhibited in a *yggS* mutant was tested in the *serA*-background. The *serA* gene encodes 3-phosphoglycerate dehydrogenase, and a *serA* mutant requires either Ser or Gly for growth. When Ser is provided, both Gly and 5,10-mTHF are generated by GlyA. In the presence of Gly, the GCV system is responsible for the production of 5, 10-mTHF, which is used for the synthesis of Ser by GlyA (Fig. 1). A *serA* mutant in which the GCV system is compromised cannot utilize Gly as a Ser source (Plamann et al. 1983). Therefore, we can assess the function of the GCV system *in vivo* using a *serA* mutant and providing Gly and/or Ser supplementation (see Fig. 1).

Growth of *serA* and *serA yggS* mutants was indistinguishable in the M9-glucose medium supplemented with 2 mM Ser (M9-Ser medium), (Fig. 4A). When grown in the M9-glucose medium supplemented with 2 mM Gly (M9-Gly medium), the growth of the *serA yggS* double mutant was significantly compromised compared to the *serA* mutant (Fig. 4B). The addition of Ser (1 mM) to the M9-Gly medium completely restored the growth of the *serA yggS* double mutant, indicating that Ser limitation caused the growth defect (data not shown). The *yggS* mutation did not affect the incorporation of Gly, since a *yggS* single mutant was able to use Gly as the sole nitrogen source (data not shown). These data supported the hypothesis that the GCV system is compromised in the *yggS* mutant.

To obtain further insight into the *yggS* mutation on the cellular metabolism, intracellular amino acid compositions in the two strains were investigated. The levels of many amino acids were altered significantly when grown in the M9-Gly medium (Fig. 4C, 4D). Concentrations of 2-AB, GABA, ophthalmic acid (OA), Ile, Met, Gly, Thr, His, and Glu

were significantly elevated in the *serA yggS* double mutant, whereas those of Ala, Asp, and Leu were slightly decreased (Fig. 4D). Many of the amino acids changed significantly were the same ones previously reported (Ito et al. 2013; Ito et al., 2016; Ito et al., 2019), suggested the perturbation of Thr/Ile/Val metabolic pathways in the *serA yggS* mutant.

GCV activities in the cell-free extract of *serA* and *serA yggS* strains grown in the M9-Gly medium were measured (Table 1). Unexpectedly, the GCV activity in the cell-free extract of *serA yggS* strain was approximately 3-times higher than in the *serA* mutant (1.2 ± 0.1, 0.4 ± 0.1 nmol/min/mg, respectively). Similar experiments were performed with the cell-free extract of *serA* and *serA yggS* strains grown in the M9-Ser medium. The GCV activity for the *serA yggS* strain was approximately 6-times higher than that for the *serA* mutant (0.31 ± 0.02 nmol/min/mg, 0.05 ± 0.01 nmol/min/mg, respectively). These data eliminated the simple possibility that *yggS* mutation lowered the expression of some of the GCV components to cause the growth defect of the *serA yggS* mutant. The reason for the increased GCV activity in the *serA yggS* mutant is unknown. GlyA activities in these two strains on M9-Gly were not significantly different (*serA*; 0.22 ± 0.01 nmol/min/mg protein, *serA yggS*; 0.20 ± 0.01 nmol/min/mg protein).

## Perturbation of $B_6$ vitamer pool correlates with growth defect of *serA yggS* mutants

A caveat of *in vitro* enzyme assays is that the cell breakage can eliminate allosteric, or competitive inhibition of enzymes due to dilution of relevant metabolites. Significantly, the YggS/Ybl036c/PLPBP family has been implicated in vitamin $B_6$ homeostasis (Darin et al., 2016; Prunetti et al., 2016; Johnstone et al., 2019; Ito et al., 2019), and the P-protein of GCV (GcvP) is a PLP-dependent enzyme (Kikuchi et al., 2008). We found that exogenous PN (> 1 μM) significantly inhibited the growth of *serA yggS* double mutant in the M9-Gly medium, but not in the presence of Ser (Fig. 5A). The growth of *serA* strain was not affected by exogenous PN on either M9-Ser or M9-Gly media (data not shown). It was possible that altered $B_6$ availability affected the GCV activity *in vivo*.

The concentration of total $B_6$ vitamers in *serA* and *serA yggS* mutants grown with Ser or Gly were analyzed (Table 2). When grown in the M9-Gly medium the *serA yggS* mutant accumulated approximately 7-times more PNP (30 ± 5.8 μM) than the *serA* mutant (6.9 ± 0.2 μM) in the cells. The elevated PNP was eliminated by plasmid-borne expression of YggS (by pUS) in the *serA yggS* mutant. High-levels of PNP was also detected when the *serA yggS* mutant was grown in the M9-Ser medium (*serA*: 5.9 ± 0.4 μM, *serAyggS*: 34.4 ± 0.7 μM). We also quantified the levels of $B_6$ vitamers in the *glyA* and *glyA yggS* double mutant. The *glyA yggS* double mutant also exhibited elevated levels of PNP in the cells (Table 2). We have shown that exogenous PN significantly increases the intracellular concentration of PNP in the *yggS* mutant (Ito et al. 2019). The PNP content in the *serA yggS* mutant grown in the M9-Ser medium supplemented with 1 μM PN was 25% higher than when grown in the absence of PN (Fig. 5B). The GCV system is a multi-enzyme complex composed of P-protein (GcvP, glycine dehydrogenase), T-protein (GcvT, aminomethyltransferase), L-protein (Lpd, lipoamide dehydrogenase), and H-protein (GcvH, lipoprotein), and the GcvP protein requires PLP for the function. These data led to hypothesize that PNP inhibits the GCV system *in vivo*.

### PNP inhibits the GCV system *in vivo*

The occurrence of the PNP-dependent inhibition of the GCV system was investigated by eliminating the accumulation of PNP with PNP/PMP oxidase (PdxH). The overexpression of the *pdxH* significantly restored the growth defect of the *serA yggS* mutant (Fig. 6A and 6B). We found that the overexpression of *gcvT-gcvH-gcvP* operon also supported the growth of *serA yggS* double mutants in the M9-Gly medium. Further, the expression of only *gcvP* alleviated the growth defect of *serA yggS* mutants (Fig. 6C and 6D). These data supported the idea that the high-levels of PNP disrupt the function of the GCV system by inhibiting the GcvP function *in vivo*.

It was formally possible that another factor(s) affected by *yggS* mutation inhibits the GCV system. To eliminate this possibility, we constructed a *serA pdxH* double mutant. Mutation of *pdxH* prevents further catabolism of PNP and induces accumulation of PNP under the *yggS*$^{+}$ background (Table 2). If the growth defect of the *serA yggS* strain was due to the accumulation of PNP, the *serA pdxH* double mutant was predicted to elicit a similar phenotype to the *serA yggS* mutant. When grown in the M9-Ser medium supplemented with 10 μM PL, the *serA* and *serA pdxH* (*yggS*$^{+}$) strains had indistinguishable growth rates (Fig. 6E). In contrast, when grown in the M9-Gly medium supplemented with 10 μM PL, the *serA pdxH* double mutant did not grow (Fig. 6F). These phenotypic data are consistent with the idea of the PNP-dependent- and YggS-independent inhibition of the GCV system.

### PNP inhibits the GCV system *in vitro*

The data shown in Table 2 represent the total intracellular concentration of $B_6$ vitamers including that which are bound to enzymes and do not reflect the concentrations of free PLP or PNP available in the cells. We thus estimated the intracellular concentrations of free $B_6$ vitamers in the *serA* and *serA yggS* mutants. We quantified the $B_6$ levels presented in the protein-free fraction, which was obtained by passing through the cell-free supernatant using a centrifugal filter device with the molecular wight cut-off of 10 kDa. This experiment found that most of the intracellular PLP (~70%) present as protein-binding form, whereas most of the PNP and PMP (~70%) exist as free-form (Fig. 7). This difference probably reflects the fact that PLP forms Schiff-base in proteins. These data indicate that the concentration of free PNP (estimated to be approximately 21 μM) is comparable to that of free PLP in the *serA yggS* mutant (Fig. 7).

In the GCV system, GcvP protein catalyzes the decarboxylation of Gly in a PLP-dependent manner. We hypothesized that PNP competes with PNP in GcvP protein and inhibits the GCV system. To test this possibility, GCV activity was measured in crude extracts with added PLP (5 μM) and varying concentrations of PNP from 0 – 250 μM. PNP reduced the GCV activity in a dose-dependent manner (Fig. 8A). In the presence of 50 μM PNP, activity was decreased by 50% at 250 μM PNP by 73%. The half-maximal inhibitory concentration ($IC_{50}$) under the condition was calculated as 59 ± 2 μM. We performed similar assays in the presence of 50 μM PLP. The experiments found that the GCV activity was inhibited by 24% at 50 μM PNP and 38% at 250 μM PNP ($IC_{50}$ > 400 μM) (Fig. 8B). These data demonstrated that PNP inhibits the GCV system by competing with PLP for binding to the enzyme. Considering the estimated free PNP level (21 μM) and the $IC_{50}$ value (59 μM), we

speculated that this inhibition may be more pronounced *in vivo*. Little GCV activity was detected when assays were performed in the absence of added PLP, suggesting that the PLP was not strongly bound to the GCV complex.

The ability of PNP to inhibit GlyA activity was also tested. Unlike the GCV system, GlyA activity was not inhibited by PNP (Fig. 8C). The slight activation observed in the presence of 250 μM PNP may be due to the contamination of PLP. The PNP stock used in this experiment had contaminating (~2%) PLP. Some other PLP-dependent enzymes including threonine synthase (ThrC), threonine dehydratase (IlvA), and branched-chain transaminase (IlvE) are also PNP insensitive (Ito et al., 2019). As shown in Table 2, the intracellular PNP concentrations in the *serA yggS* strain were not significantly affected by the extracellular amino acid (M9+Ser: 30 μM and M9+Gly: 34 μM). In contrast, the amino acid pool of *serA yggS* strain was significantly perturbed only in the M9+Gly medium (Fig. 4C and 4D). The GCV reaction is essential for the growth in the M9+Gly medium but dispensable in the M9+Ser medium. Therefore, we can estimate that the GCV system is the most PNP-sensitive target in *E. coli* under the condition examined. Wild-type *E. coli* cells produce most of their one-carbon units required by the GlyA reaction, and the GCV system plays a minor role (Meedel et al., 1974). This fact may explain the milder phenotypes of the *yggS* mutants observed under the $glyA^+$ background.

GCV system is highly conserved in diverse organisms ranging from bacteria to humans, where it plays an important role in the degradation of Gly as well as one-carbon unit metabolism. GcvP of *E. coli*, a PLP-dependent component of the GCV system, exhibits approximately 50% sequence identity to the corresponding protein of human and yeast protein. We found that the GCV system of *Bacillus subtilis*, whose glycine decarboxylase (P-protein, subunit 1 and subunit 2) shows more than 40% identity to that of *E. coli* GcvP, is weakly inhibited by PNP (Fig.5D). Although PNP is not known to accumulate in *B. subtilis*, this result suggested the PNP-sensitive feature of the GCV system. The PLPBP-deficient human cells (fibroblasts and HEK293 cells) accumulate a considerable amount of PNP (Johnstone et al., 2019). The PLPBP-deficient strain of *Saccharomyces cerevisiae* also accumulates high-levels of PNP in the cells (Vu et al, unpublished). It is interesting to examine the occurrence of PNP-dependent inhibition of the GCV system and/or other PLP-dependent enzymes in the PLPBP-deficient organisms.

## Working model for diverse phenotypes observed in the *yggS* mutant

The *yggS* mutant accumulates some metabolites in the Thr/Ile/Val metabolic pathway (Val, ketoisovalerate (KIV), Ile, 2-AB, and OA) and exhibits decreased level of coenzyme A (Ito et al., 2013; Ito et al. 2016; Ito et al., 2018). Some of these changes correlate with increased levels of PNP, but the mechanism was unknown (Ito et al., 2019). The present findings may explain the mechanism of diverse phenotypes observed in the *yggS* mutant as described below and shown in Fig. 8.

The *yggS* mutation induces PNP accumulation by an unidentified mechanism. The high-levels of PNP inhibit the GCV system and decrease cellular 5,10-mTHF levels. The limitation of 5,10-mTHF would lower the activity of 3-methyl-2-oxobutanoate hydroxymethyltransferase (PanB), which catalyzes the conversion of the 5,10-mTHF and

KIV to form 2-dehydropantoate and THF (Teller et al., 1976; Powers et al., 1976). It can decrease the production of the CoA precursor 2-dehydropantoate and increase the KIV (and its transamination product Val) in the cells. Val activates threonine dehydratase (IlvA) and inhibits acetohydroxy acid synthases (AHAS I/III) to produce more 2-ketobutyrate (Leavitt et al., 1962; Eisenstein, 1991). 2-ketobutyrate is the precursor of Ile, 2-AB, and OA (Fig. 7).

## Conclusion remarks

We demonstrated that high-levels of PNP induced by *yggS* mutation cause the conditional lethality in the *glyA*- *or serA*-background by inhibiting the GCV system. The PNP-dependent inhibition of the GCV system can provide a plausible reason for the diverse phenotypes observed in *E. coli*. Further investigations focusing on the metabolism of PNP will shed light on the molecular function of the YggS/Ybl036c/PLPBP family and the link between the perturbation of $B_6$ pools and diverse metabolic pathways.

## Experimental procedures

### Bacterial strains and culture conditions.

The strains used in this study are listed in Table 3. When added to the medium, ampicillin (Amp), chloramphenicol (Cm) and kanamycin (Km) were used at concentrations of 100 μg/ml, 30 μg/ml, and 50 μg/ml, respectively. Single-gene deletion mutants of *E. coli* (Keio collection) and folD-expression plasmid (pCA24N-folD, ASKA clone) were obtained from NBRP (Baba et al., 2006; Kitagawa et al., 2005). *E. coli* strains were grown in an LB medium or M9-glucose medium as previously described (Ito et al. 2013; Ito et al. 2019). Ser, Gly, or Met were added at a final concentration of 2 mM. Casamino acid or nucleotide was supplemented at a final concentration of 0.4% or 0.2 mM, respectively. Unless otherwise noted, *E. coli* strains were grown at 30°C. Cells growth was recorded by the OD-Monitor C&T apparatus (Taitec Co., Ltd., Koshigaya, Japan) using glass test tubes (16.5 mm in diameter by 165 mm in height), or the ELx808 (Biotec, Winooski, VT, USA) using 96-wells plate.

### Molecular genetics and sequence analysis.

The deletion of the *yggS* gene was performed using the bacteriophage λ-Red recombinase system described by Datsenko and Wanner (Datsenko et al., 2000). The *glyA yggS* double mutant was constructed as follows. An *E. coli glyA* mutant (Keio collection, JW2535-KC) harboring pCP20 (Cherepanov et al., 1995) was streaked on an LB plate and grown at 42°C, forming $glyA^{-Km}$ strain. A PCR product was generated with primers yggS-H1 and yggS-H2 (Ito et al., 2009) using Tks Gflex DNA polymerase (TaKaRa) and pKD13 as a template. The PCR product was purified from agarose-gel and electroporated into the $glyA^{-Km}$ harboring pKD46. Resultant transformants appeared on an LB plate containing 1 mM Met, 0.1 mM thymidine, and 30 μg/ml kanamycin were screened by PCR for the appropriate insertion of the kanamycin-resistant gene with the primers yggS-200up and yggS-300dwn (Ito et al., 2009). Construction of the *serA yggS* double mutant and *serA pdxH* double mutant was performed in a similar way using the JW2880-KC (Keio collection) as parental strains, respectively. Primers pair of pdxH-H1 (5′-

ATGTCTGATAACGACGAATTGCAGCAAATCGCGCATCTGCGCCGTGAATGTGTAG GCTGGAGCTGCTTCG-3′) and pdxH-H2 (5′-TCAGGGTGCAAGACGATCAATCTTCCACGCATCATTTTCACGCTGGTCATATGAAT ATCCTCCTTAG-3′) were used for the construction of *pdxH*-deficient strain. Insertion of kanamycin-resistant cassette at the *pdxH* locus was confirmed with the primers pdxH-100up (5′-CGCATCGTCTTGAATAACTGTCAG-3′) and pdxH-100dwn (5′-CACCTTTGCCGGTACACGACTTTTC-3′). The *gcv* operon (*gcvT-gcvH-gcvP*) or *gcvP* gene was amplified with the primers gcvT-fw (5′-GCTAACAGGAGGAATTAACCATGGCACAACAGACTCCTTTGTACG-3′) or gcvP-fw (5′-GGCTAACAGGAGGAATTAACCATGACACAGACGTTAAGCCAGCTTG-3′) and gcvP-rv (5′-GATGAGTTTTTGTTCTACGTCGCCGAAGCGCCTTTAGAAAATAG-3′) and cloned into pBAD/MycHisC plasmid (Invitrogen) using In-Fusion HD Cloning Kit (Takara) according to the manufacture's instruction.

## GCV assay

GCV activity was assayed as described previously with some modifications (Meedel et al., 1974; Nagarajan et al., 1997). *E. coli* cells (*serA* or *serA yggS* strains) were grown in an M9-glucose medium supplemented with 1 mM Ser or 1 mM Gly. To examine the effect of PNP to GCV system, a cell-free extract of *glyA*-deficient strain grown in M9-glucose medium supplemented with 1 mM Gly was used. The *gcvP*-deficient *E. coli* was used as a negative control. The cells pellet (500 mg) was sonicated in 5 ml of phosphate buffer (20 mM potassium phosphate buffer (pH 7.4), 10% glycerol) and centrifuged (20,000 g, 30 min, 4°C). The protein concentrations in the cell-free extract were determined using the BioRad Bradford assay (BioRad). The reaction was initiated by mixing 125 μl of the cell-free extract and 125 μl of a reaction mixture containing 40 mM potassium buffer (pH 7.4), 4 mM dithiothreitol (DTT), 2 mM tetrahydrofolate (THF), 5 μM or 50 μM PLP, 0 to 250 μM PNP, 4 mM nicotinamide adenine dinucleotide ($NAD^+$), and 1.2 nmol [2-$^{14}$C] Gly (53.0 mCi / mmol). After 2 hours of incubation at 30°C, 250 μl of a solution (a mixture of 150 μl of 1 M sodium acetate (pH 4.5) and 100 μl of 0.1 M formaldehyde) and then 150 μl of 0.4 M dimedon in 50% ethanol was added to the reaction mixture. After 5 min incubation at 65°C, 2.5 ml of toluene was added and vortexed vigorously for 1 minute. After centrifugation, the radioactivity of the toluene layer (2 ml) was measured by liquid scintillation counting using AccuFLEX LSC-7200 (Hitachi Aloka Medical Co., Ltd.). PNP was synthesized by reduction of PLP with $NaBH_4$, and purified as described previously (Argoudelis et al. 1986).

## GlyA assay

GlyA activity in the cell-free extract of *serA* or *serA yggS* double mutant was assayed according to the protocol described previously with some modifications (Schirch et al., 1968). Cell-free extracts of *serA* or *serA yggS* double mutant were prepared as described above. Reaction was initiated by adding 10 μl of the cell-free extract to the 890 μl of reaction mixture containing 50 mM Hepes-NaOH (pH 7.5), 0.4 mM THF, 0.2 mM $NADP^+$, 5 mM Ser, 5 μM PLP, and 0.1% 2-mercaptoethanol. After 20 min incubation at 37°C, 100 μl of 0.1 M potassium carbonate buffer (pH 9.5) was added to terminate the reaction. Then 20 μl of the purified His-tagged *E. coli* 5, 10-methylene tetrahydrofolate dehydrogenase (FolD, 5

mg/ml) was added, and the absorbance change at 340 nm was recorded. The recombinant FolD was prepared from an *E. coli* AG1 strain harboring pCA24N-folD by His-tag affinity chromatography using a conventional method.

## Amino acid and $B_6$ vitamer analysis

The *glyA* or *glyA yggS* double mutants were grown in an M9 medium supplemented with 0.4% casamino acid. The *serA* or *serA yggS* double mutants were grown in an M9 medium containing 1 mM Ser or 1 mM Gly. The log-phase cells ($OD_{600}$ of 0.4 – 0.6) were collected by centrifugation. For the quantification of total $B_6$ content, the cell pellet was resuspended in 10 volumes (v/w) of 0.8 M $HClO_4$ (100 μl of the $HClO_4$ solution for 10 mg *E. coli* wet cells). The suspension was vortexed and incubated for 30 min at 4°C. 5 volumes (v/w) of 0.8 M $K_2CO_3$ solution (50 μl for 10 mg *E. coli* cells) was added. Debris was removed by centrifugation (20,000 × *g* for 20 min at 4°C). For the analyses of "free $B_6$" ($B_6$ vitamer that is not tightly bound to large-molecule), the cells pellet was sonicated in 5 volumes of 50 mM Hepes-NaOH buffer (pH 7.5). The cell-free supernatant was obtained by centrifugation (20,000 g, 30 min). Protein concentration in the soluble fraction was determined with the BioRad protein assay kit (BioRad) using bovine serum albumin as standard. The protein-free solution was prepared by passing the cell-free supernatant through the Amicon-ultra centrifugal membranes with 10 kDa molecular weight cutoff. These solutions (180 μl) were deproteinized with $HClO_4$ (8 M, 20 μl) and neutralized with 100 μl of the 0.8 M $K_2CO_3$. After centrifugation, samples were diluted and used for the HPLC analyses as previously described (Ito et al., 2018; Ito et al., 2019). Previous results reported that 1 mg of wet cell weight corresponds to 0.23 mg of dry cell weight, and the amount of cytoplasmic water in cells grown in the M9-glucose is 2.0 μl/mg dry cell weight (Glazyrina et al. 2010, Cayley et al. 1991). We thus estimated that 1 pmol/mg wet cells of PLP correspond to 2.17 μM of intracellular PLP.

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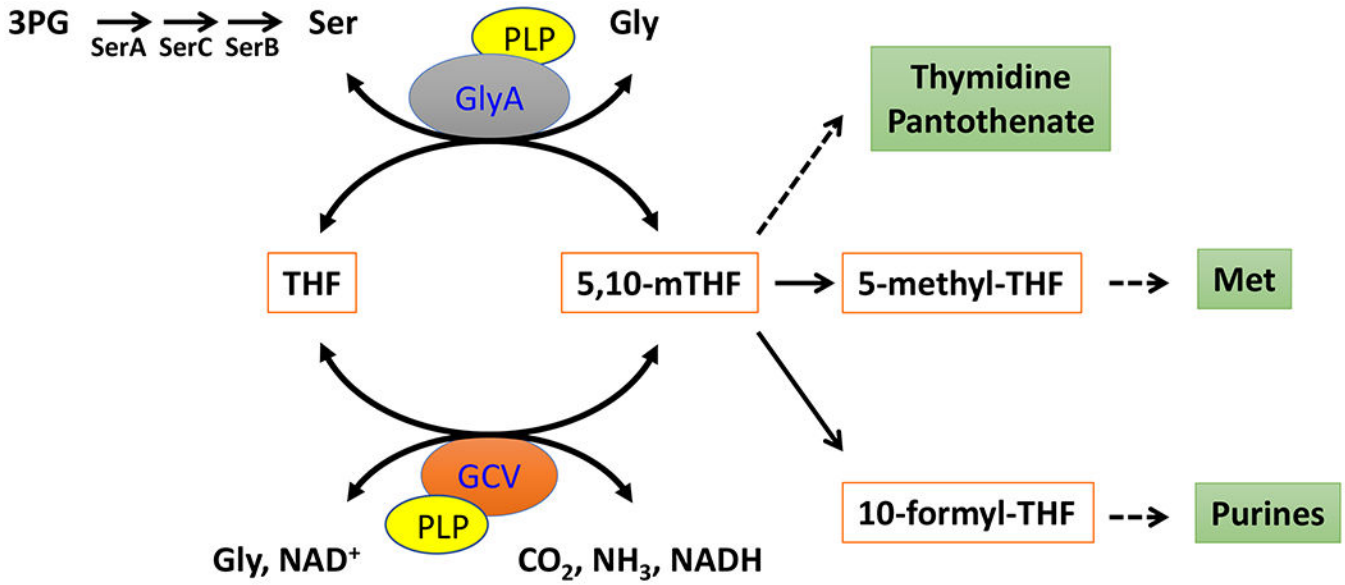


**Figure 1. Reaction of GlyA and GCV system**

GlyA is PLP-dependent enzyme and catalyzes conversion of Ser to Gly, while transferring the hydroxymethyl group to tetrahydrofolate (THF), and generates 5, 10-methyl-tetrahydrofolate (5,10-mTHF). GCV system cleaves Gly to $CO_2$, ammonia and provides 5,10-mTHF. GlyA and GCV reactions require PLP. In the wild-type *E. coli*, GlyA can provide most of the 5,10-mTHF (Meedele et al., 1974). In the absence of *glyA*, GCV system provides 5,10-mTHF for one-carbon biosynthesis.

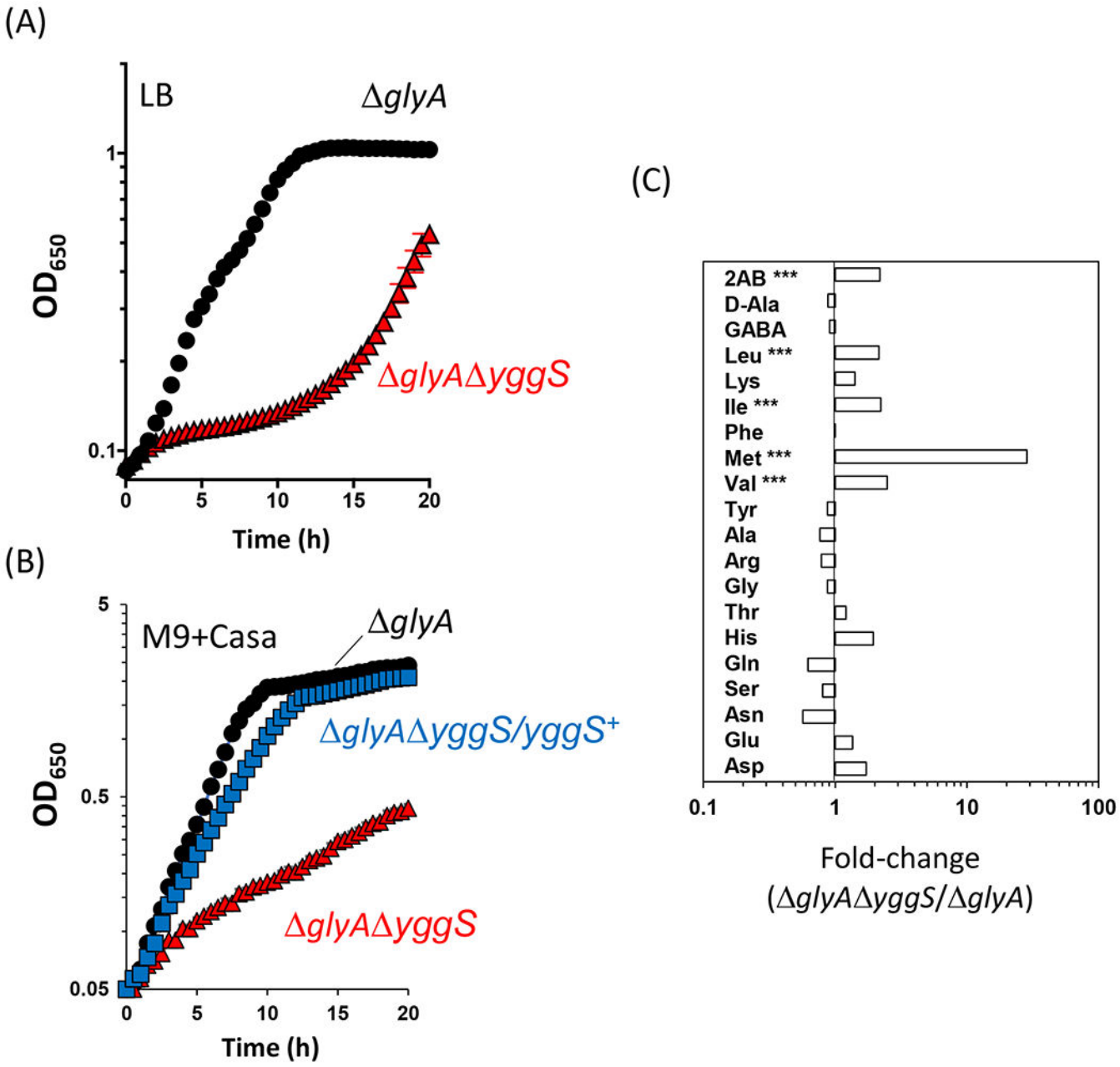


**Figure 2. Effect of *yggS* mutation under *glyA* background on the growths and intracellular amino acid pool**

(A) Growth of the *glyA* and *glyA yggS* mutants in the LB medium. (B) Growth of the *glyA* strain and *glyA yggS* mutants both harboring pU0 plasmid (pUC19 containing partial sequence of *yggS*) and *glyA yggS* mutant harboring pUS plasmid (yggS expression vector) (Ito et al. 2009) in the M9-Casa medium. Cells growth was recorded by the ELx808. (C) Intracellular amino acid pool of *glyA* or *glyA yggS* mutants grown in the M9-Casa medium. Amino acid pools were analyzed as described in Experimental procedure. Disruption of *yggS* under *glyA* background affects Ile/Val and Met metabolisms. (*p < 0.05, **p < 0.01, ***p <0.001, *P < 0.05, **P < 0.01, student's t-test)

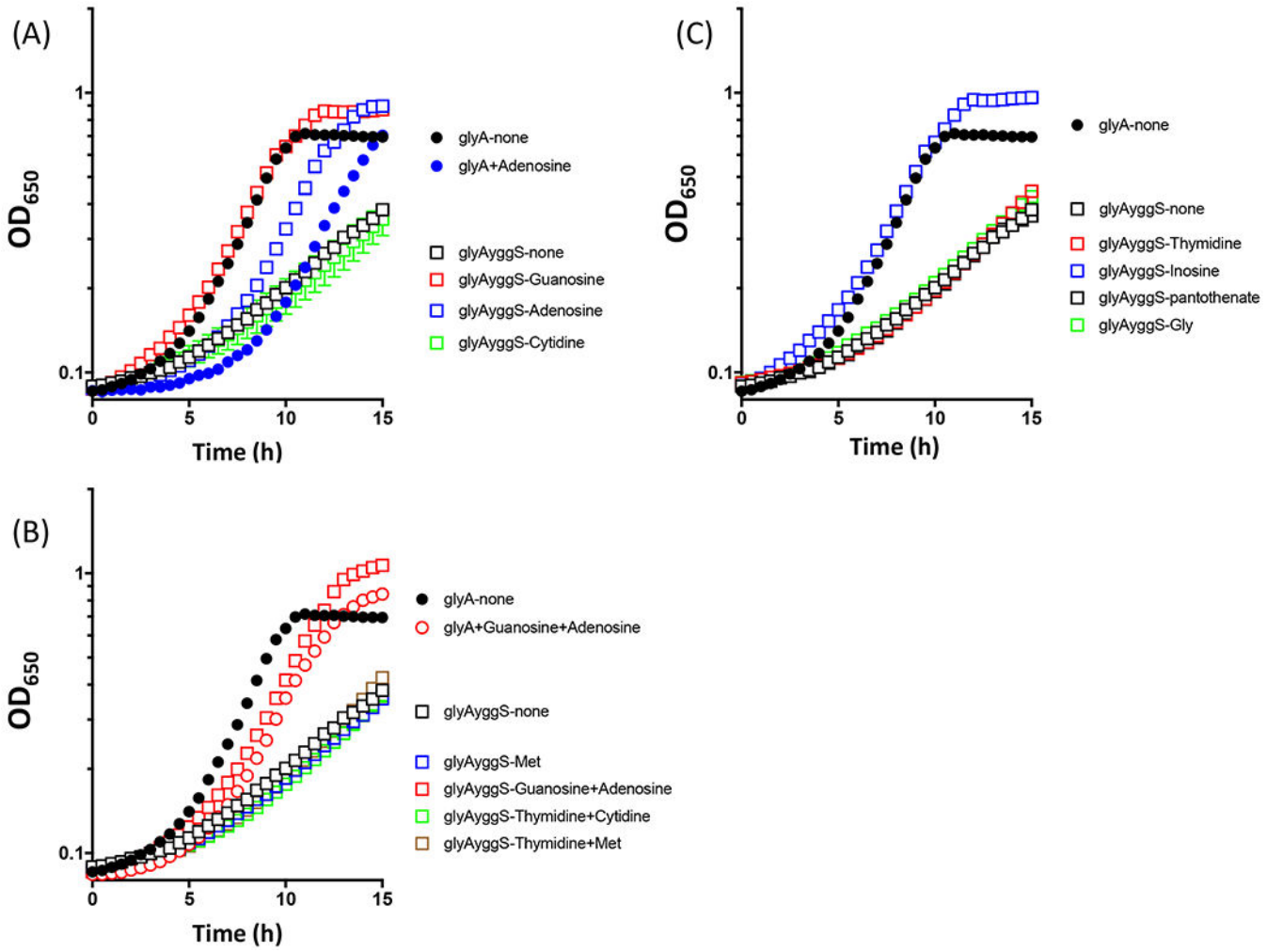


**Fig. 3. Effect of nucleotide and/or amino acid on the growth of *glyA yggS***

Growths of the *glyA* (circle) and *glyA yggS* double mutant (square) in the M9-Casa medium in the presence of nucleotide and/or amino acid. The concentration of nucleotide or amino acid (Met or Gly) was 0.2 mM or 2 mM, respectively. Guanosine (A), adenosine (B), or inosine (C) supported the growth of *glyA yggS* double mutant. Adenine inhibited the growth of the *glyA* (A). Other nucleotides and amino acids did not significantly affect the growth of *glyA* strain (data not shown). The data represent the averages and standard deviations from triplicate experiments. Cells growth was monitored by the ELx808.

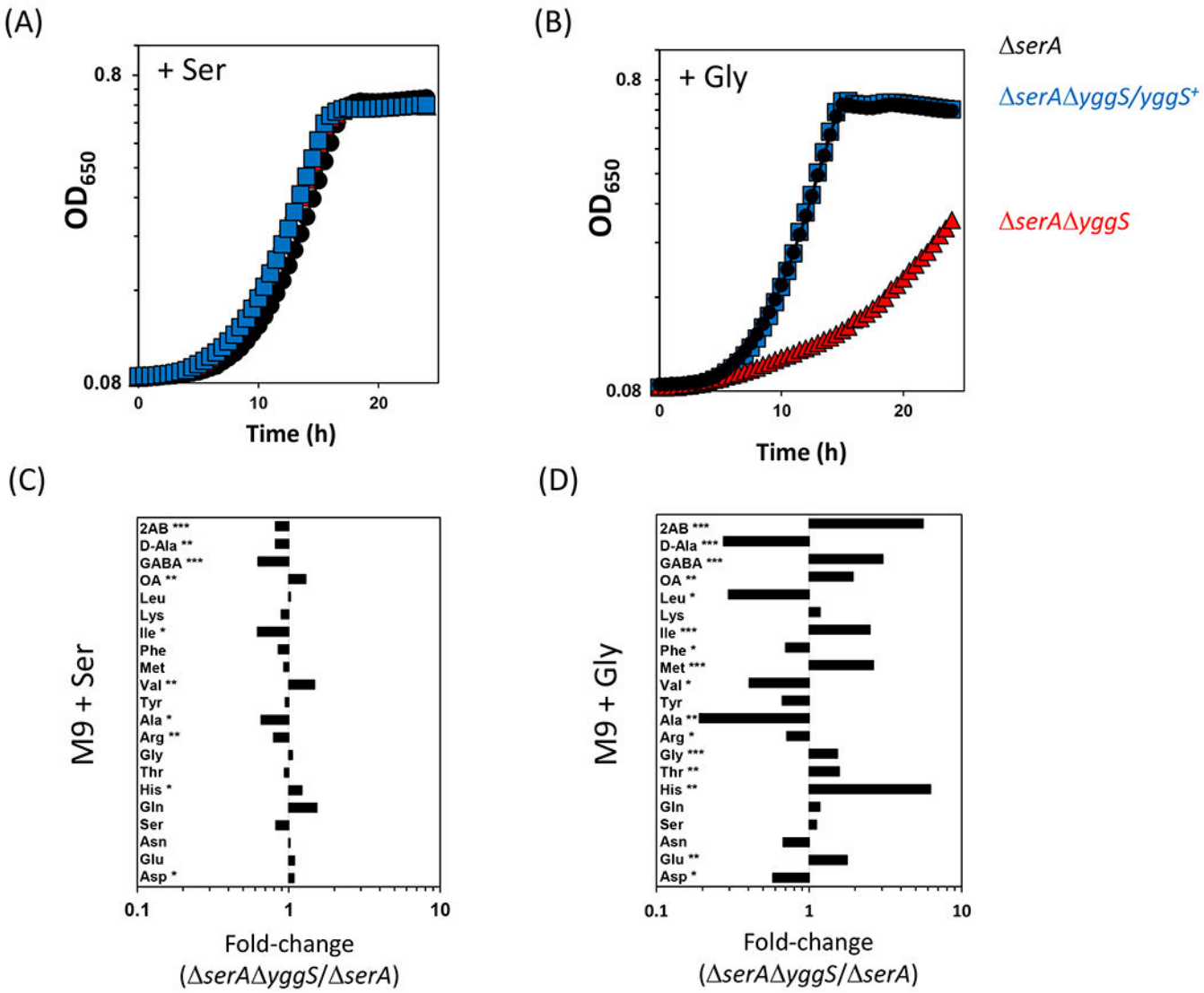


**Fig. 4. Effect of *yggS* mutation under *serA* background on the growths and intracellular amino acid pool**

(A, B) Growths of the *serA* and *serA yggS* double mutant in the (A) M9-Ser or (B) M9-Gly medium. When grown in the M9-Ser medium, the two strains exhibited almost identical growth. When grown in the M9-Gly medium, the *serA yggS* double mutant showed poor growth. Cells growth was recorded by the ELx808. (C, D) Differences of amino acid pools of *serA* and *serA yggS* grown in the (C) M9-Ser or (D) M9-Gly medium. When grown in the M9-Gly medium, the amino acid pool of *serA yggS* strain was significantly different from that of *serA* strain. Experiments were performed in triplicate, and data are represented as the fold-change. (*p < 0.05, **p < 0.01, ***p <0.001, *P < 0.05, **P < 0.01, student's t-test)

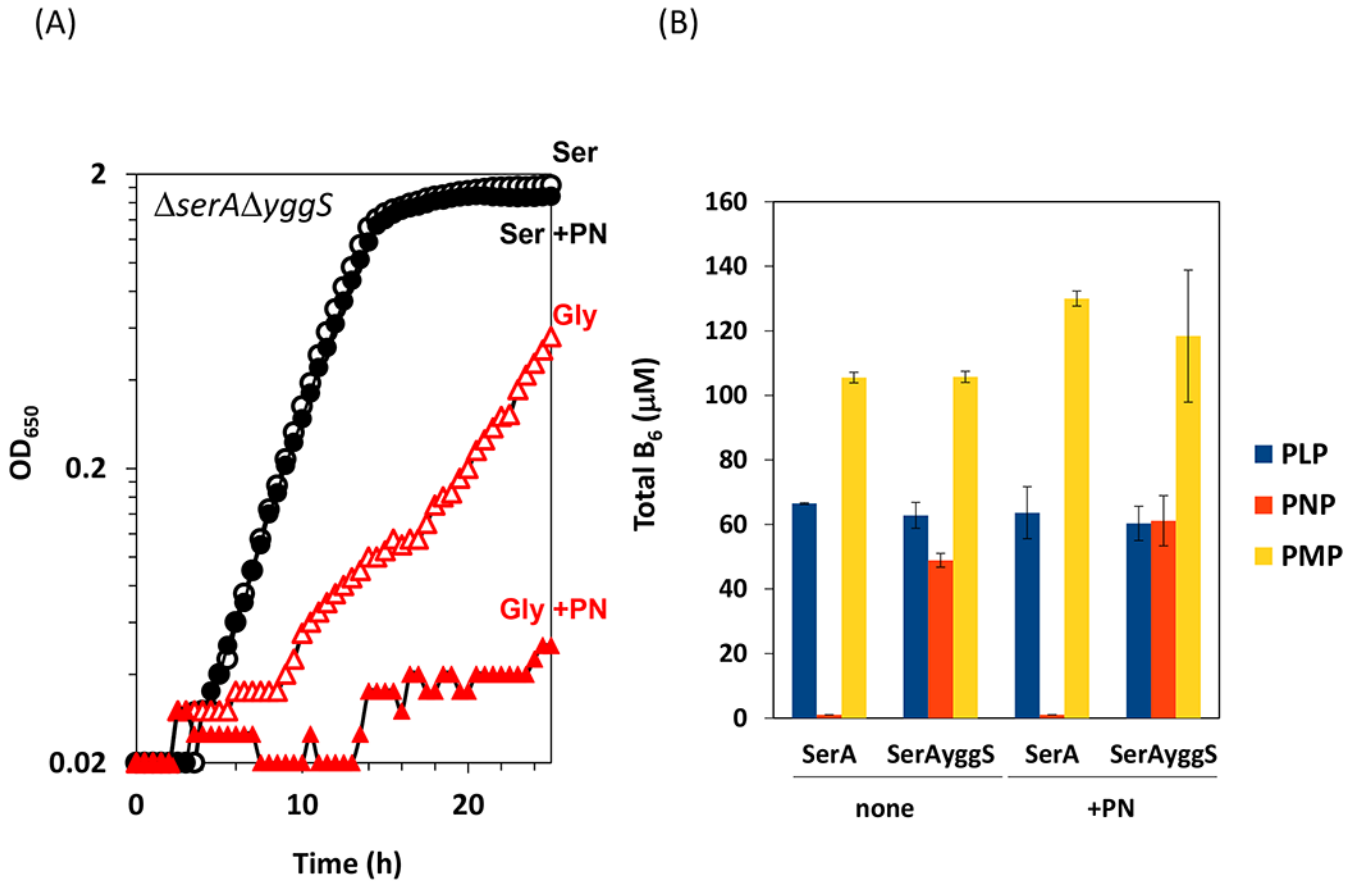


**Fig. 5. Effect of PN on the growths and concentrations of total $B_6$ vitamers of *serA yggS* strain**

(A) Growths of the *serA yggS* double mutant in the M9-Ser (black) or M9-Gly medium (red) in the presence (open symbol) or absence of PN (1 μM) (closed symbol). When grown in the M9-Gly medium, the growth of *serA yggS* double mutant was further inhibited by PN. Cells growth was recorded by the OD-Monitor C&T apparatus. (B) Concentrations of $B_6$ vitamers in *serA yggS* mutant grown in the absence or presence of 1 μM of PN. Exogenous PN significantly increased the intracellular content of PNP.

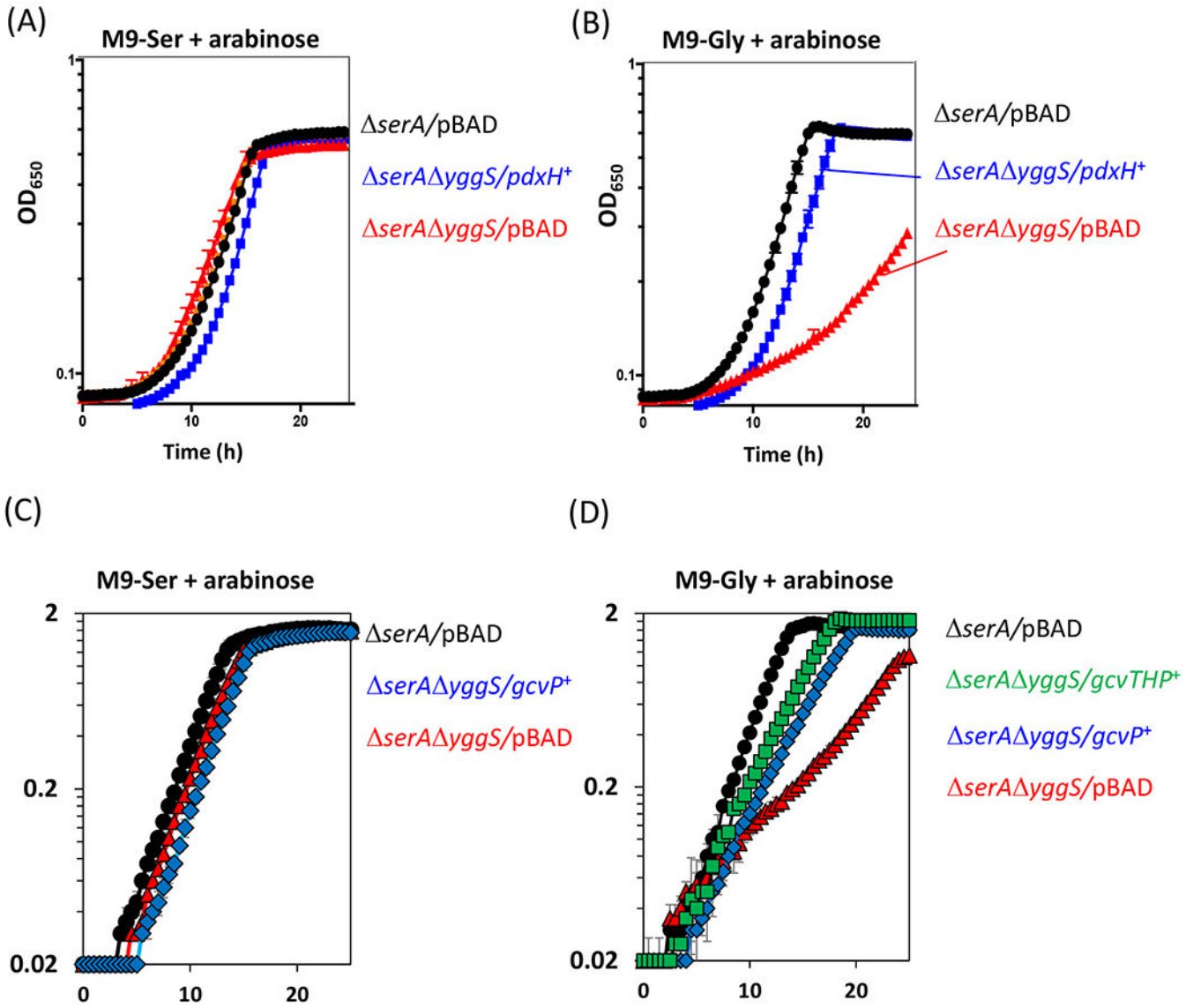


**Fig. 6. PNP inhibits GCV system in vivo**

(A, B, C, D) Growths of *serA* and *serA yggS* double mutant harboring pBAD empty vector (pBAD), pBAD-pdxH, pBAD-gcvTHP (*gcvTHP*$^+$), or pBAD-gcvP (*gcvP*$^+$) plasmid (*pdxH*$^+$) were compared in the M9+Ser or M9+Gly medium. Expression *of gcvT-gcvH-gcvP* (*gcvTHP*$^+$), *gcvP*, or *pdxH* was induced by 0.2% or 0.02% arabinose, respectively. The expression of *pdxH*, *gcvT-gcvH-gcvP,* or *gcvP* significantly improved the growth of *serA yggS* double mutant in the M9-Gly medium. Note that growth of *serA* mutant strain was not significantly affected by the overexpression of *gcvP* or *pdxH*. (E, F) Growth of *serA* and *serA pdxH* double mutant in a (E) M9-Ser + 10 μM PL or (F) M9-Gly + 10 μM PL medium. The *serA pdxH* double mutant exhibited lethality in the M9-Gly + 10 μM PL medium. Cells growth was recorded by the OD-Monitor C&T apparatus (panels C, D) or the ELx808 (panels A, B, E, F) using 96-wells plate.

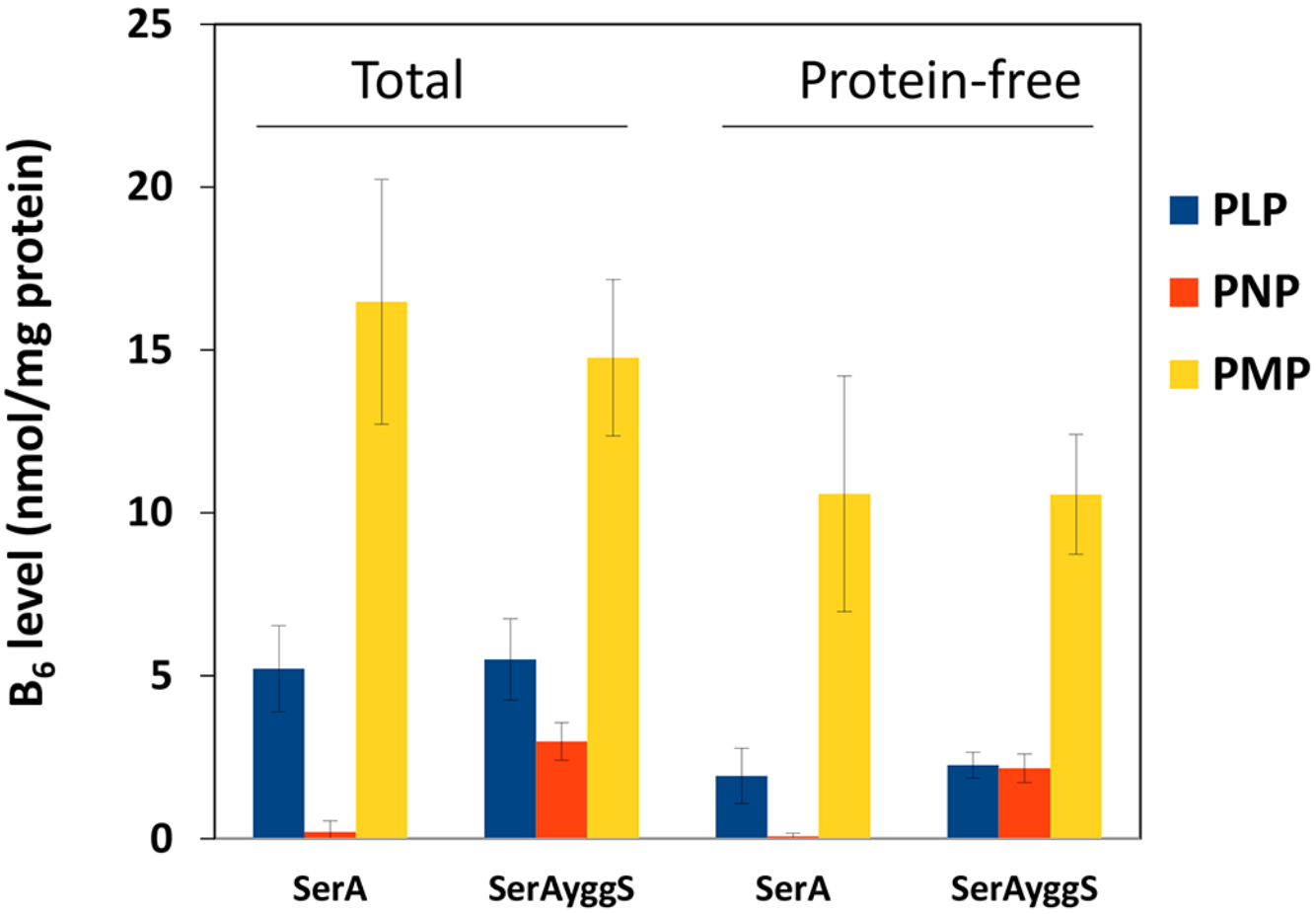


**Fig. 7. Estimation of free $B_6$ levels in the *serA yggS* mutant**

The *serA* or *serA yggS* double mutant was cultivated in the M9+Gly medium. The cells were disrupted and centrifuged. The resultant cell-free fraction was passed through the centrifugal filter device (10 kDa-cut off) and obtained the protein-free fraction. The $B_6$ levels in the cell-free fraction (total $B_6$) or the protein-free-fraction were determined as described in the Experimental procedure. In the *serA yggS* mutant, most of the PNP was presented as free-form and the concentration was almost identical to the free PLP concentration.

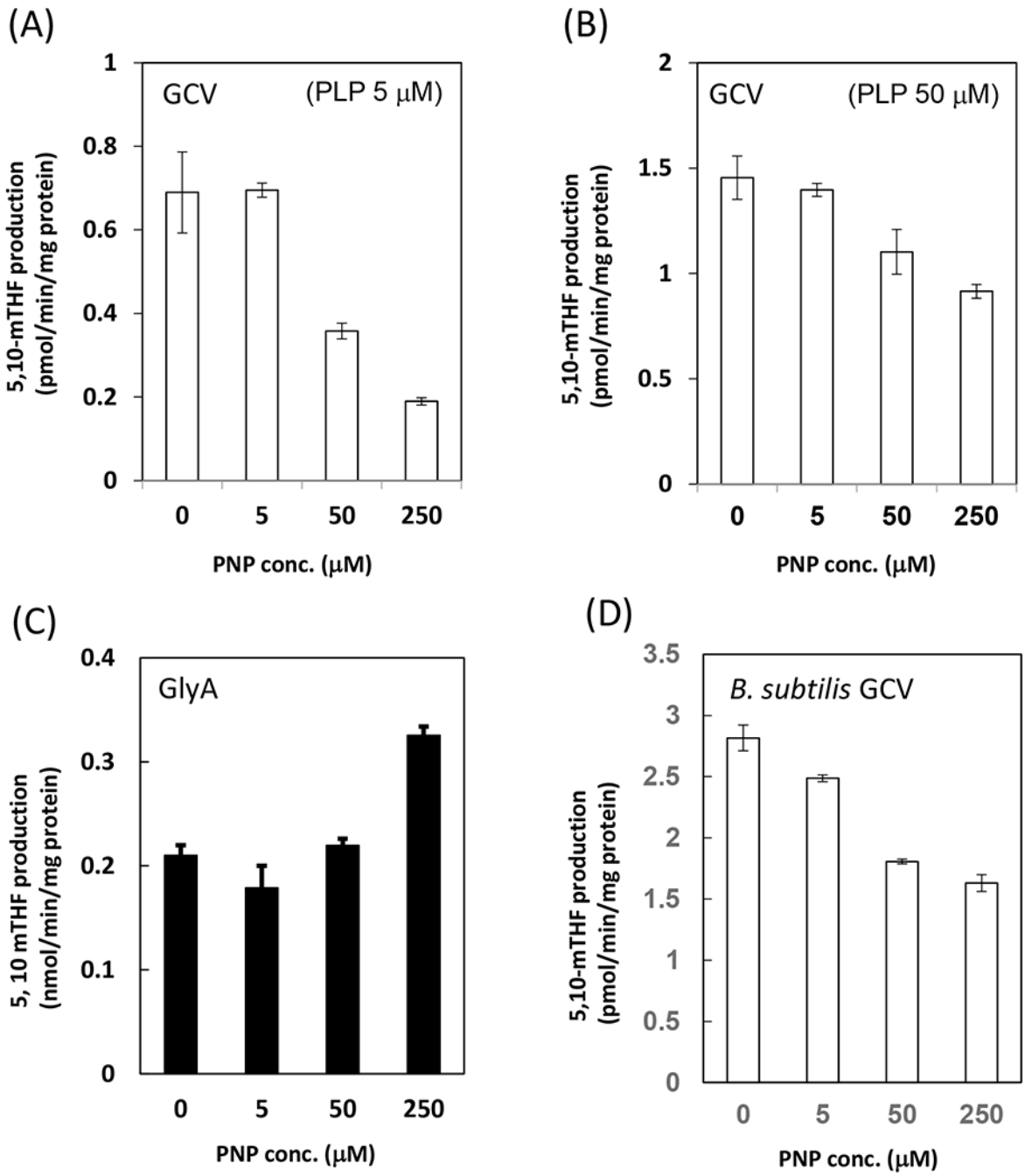


**Fig. 8. PNP inhibits GCV system *in vitro***

GCV activity was measured in the presence of (A) 5 μM or (B) 50 μM of added PLP, and various concentrations of PNP (0, 5, 50, or 250 μM). Cell-free extract of *glyA* strain grown in the M9-Gly medium was used for the analyses. No GCV activity was detected in the absence of added PLP. (C) Effect of PNP on GlyA activity was also assayed using a purified GlyA in the presence of 5 μM PLP and various concentration of PNP. (D) Effect of PNP on GCV activity of *B. subtilis* was also assayed using cell-free extract in the presence of 5 μM

PLP and various concentration of PNP. Experiments were performed in triplicate, and data represent the averages and standard deviations of the means.

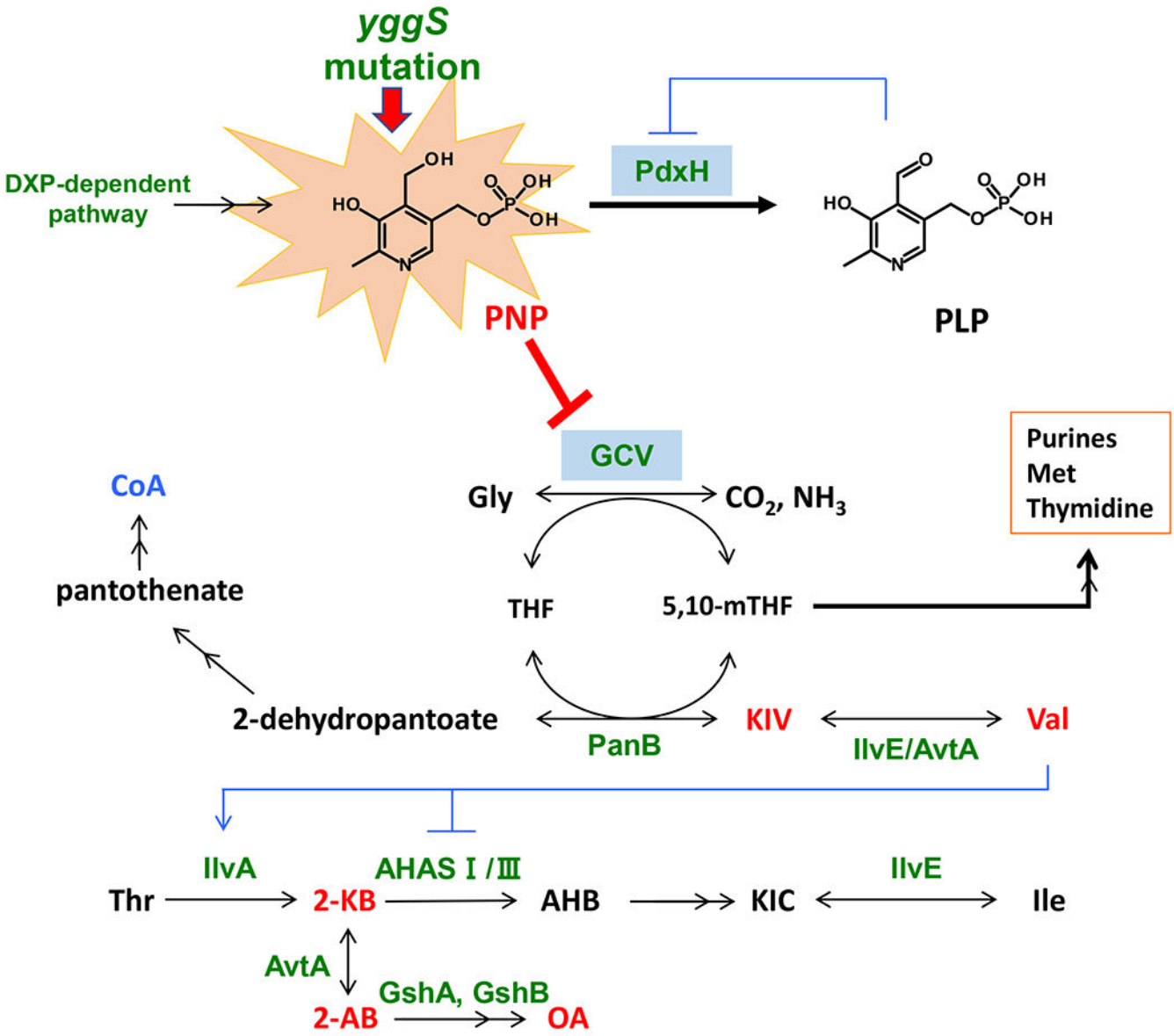


**Fig. 9. Connection of PNP, GCV system, and phenotypes observed in the *yggS*-deficient *E. coli*.**

Deletion of *yggS* in *E. coli* induces accumulation of PNP by unidentified mechanism. High-levels of PNP compete with PLP and inhibit GCV system. Disruption of GCV system can decrease 5,10-mTHF supply, which may decrease flux into pantothenate production and increase flux for Val production. Val stimulates threonine dehydratase (IlvA) to produce more 2-ketobutyrate (2-KB) as a precursor for 2-aminobutyrate (2-AB), Ile, and ophthalmic acid (OA).

## Table 1

**Activity of GCV or GlyA in the cell-free extract of *serA* and *serA yggS* strains**

The *serA* or *serA yggS* strains harboring pU0 plasmid were grown in the M9-Ser or M9-Gly medium. Ampicillin was added for plasmid maintenance. The *E. coli* strains were collected at log-phase and disrupted by sonication. The enzyme activities were analyzed as described in Experimental Procedures. The data represent the averages and standard deviations from triplicate experiments.

| | GCV activity (5, 10-mTHF production (pmol)/mg protein) | | GlyA activity (5, 10-mTHF production (pmol)/mg protein/min) | |
|---|---|---|---|---|
| | + Ser | + Gly | + Ser | + Gly |
| ***serA*** | 11 ± 1 | 69 ± 4 | 170 ± 6 | 220 ± 12 |
| ***serA yggS*** | 66 ± 1 | 215 ± 19 | 192 ± 8 | 202 ± 15 |

## Table 2

**Total vitamin $B_6$ levels in the *E. coli* strains**

The *serA* or *serA yggS* strains harboring pU0 plasmid or complementary pUS plasmid (expresses YggS protein) were grown in the M9-Ser or M9-Gly medium. The *serA pdxH* strain was grown in the M9-Ser medium in the presence of 10 μM PL. The *glyA* and *glyA yggS* strains were grown in the M9-Casa medium. Amp (100 μg/ml) was added for plasmid maintenance. The *E. coli* strains were collected at log-phase and the total $B_6$ pools were analyzed as described in Experimental Procedures. The data represent the averages and standard deviations from triplicate experiments.

| | PLP conc. (μM) | PNP conc. (μM) | PMP conc. (μM) |
|---|---|---|---|
| (M9 + Gly) | | | |
| *serA* | 57 ± 4 | 7 ± 0.2 | 146 ± 14 |
| *serA yggS* | 49 ± 10 | 32 ± 5.8 | 121 ± 24 |
| *serA yggS/yggS*$^{+}$ | 62 ± 2 | 9 ± 0.6 | 147 ± 14 |
| (M9 + Ser) | | | |
| *serA* | 68 ± 5 | 7 ± 0.4 | 145 ± 3 |
| *serA yggS* | 78 ± 4 | 38 ± 1 | 132 ± 6 |
| *serA yggS/yggS*$^{+}$ | 69 ± 3 | 6 ± 0.3 | 138 ± 6 |
| *serA pdxH* | 55.7 ± 3.6 | 159 ± 36 | 44 ± 10 |
| (M9 + Casa) | | | |
| *glyA* | 83 ± 7 | N.D.[a] | 84 ± 2 |
| *glyA yggS* | 98 ± 11 | 28 ± 1 | 127 ± 6 |

[a] N.D.: Not Detected

**Table 3**

*E. coli* strains and plasmids used in this study

| **Strains** | | |
|---|---|---|
| *glyA* | *E. coli* BW25113 *glyA*::Km (JW2535-KC) | Keio collection |
| *glyA*$^{-Km}$ | *E. coli* BW25113 *glyA* | This study |
| *glyA yggS* | *E. coli* BW25113 *glyA yggS*::Km | This study |
| *serA* | *E. coli* BW25113 *serA*::Km (JW2880-KC) | Keio collection |
| *serA*$^{-Km}$ | *E. coli* BW25113 *serA* | This study |
| *serA yggS* | *E. coli* BW25113 *serA yggS*::Km | This study |
| *serA pdxH* | *E. coli* BW25113 *serA pdxH*::Km | This study |
| *gcvP* | *E. coli* BW25113 *gcvP*::Km (JW2871-KC) | Keio collection |
| | | |
| **Plasmids** | | |
| pU0 | pUC19 containing a partial sequence of *yggS* | Ito et al., 2009 |
| pUS | pUC19 expressing *yggS* | Ito et al., 2009 |
| pBAD24 | pBAD24 empty vector | Laboratory collection |
| pBAD24-pdxH | pBAD24 containing *pdxH* from *S. enterica* | Vu et al. to be published |
| pBAD-gcvTHP | pBAD-MycHisC containing *gcvT-gcvH-gcvP* | This study |
| pBAD-gcvP | pBAD-MycHisC containing *gcvP* | This study |
| pCA24N-folD | pCA24N containing *folD* (JW0518-AM) | ASKA clone |
| pKD13 | A template plasmid for gene disruption. The Km$^r$ gene is flanked by FRT sites. | Datsenko et al. 2000 |
| pKD46 | Lambda Red recombinase expression plasmid | Datsenko et al. 2000 |
| pCP20 | Yeast Flp recombinase expression plasmid with temperature-sensitive replication. | Nagarajan et al. 1997 |