Conserved Pyridoxal 5'-Phosphate-Binding Protein YggS Impacts Amino Acid Metabolism through Pyridoxine 5'-Phosphate in *Escherichia coli*

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ABSTRACT *Escherichia coli* YggS (COG0325) is a member of the highly conserved pyridoxal 5'-phosphate (PLP)-binding protein (PLPBP) family. Recent studies suggested a role for this protein family in the homeostasis of vitamin B₆ and amino acids. The deletion or mutation of a member of this protein family causes pleiotropic effects in many organisms and is causative of vitamin B₆-dependent epilepsy in humans. To date, little has been known about the mechanism by which lack of YggS results in these diverse phenotypes. In this study, we determined that the pyridoxine (PN) sensitivity observed in *yggS*-deficient *E. coli* was caused by the pyridoxine 5'-phosphate (PNP)-dependent overproduction of Val, which is toxic to *E. coli*. The data suggest that the *yggS* mutation impacts Val accumulation by perturbing the biosynthesis of Thr from homoserine (Hse). Exogenous Hse inhibited the growth of the *yggS* mutant, caused further accumulation of PNP, and increased the levels of some intermediates in the Thr-Ile-Val metabolic pathways. Blocking the Thr biosynthetic pathway or decreasing the intracellular PNP levels abolished the perturbations of amino acid metabolism caused by the exogenous PN and Hse. Our data showed that a high concentration of intracellular PNP is the root cause of at least some of the pleiotropic phenotypes described for a *yggS* mutant of *E. coli*.

IMPORTANCE Recent studies showed that deletion or mutation of members of the YggS protein family causes pleiotropic effects in many organisms. Little is known about the causes, mechanisms, and consequences of these diverse phenotypes. It was previously shown that *yggS* mutations in *E. coli* result in the accumulation of PNP and some metabolites in the Ile/Val biosynthetic pathway. This work revealed that some exogenous stresses increase the aberrant accumulation of PNP in the *yggS* mutant. In addition, the current report provides evidence indicating that some, but not all, of the phenotypes of the *yggS* mutant in *E. coli* are due to the elevated PNP level. These results will contribute to continuing efforts to determine the molecular functions of the members of the YggS protein family.

KEYWORDS PLPBP, PROSC, pyridoxal 5'-phosphate, pyridoxine 5'-phosphate, YggS, amino acid biosynthesis, vitamin B₆

Escherichia coli YggS (COG0325) is a member of the highly conserved pyridoxal 5'-phosphate (PLP)-binding protein (PLPBP) family whose biochemical function is unknown (1–3). The YggS protein family is present in all three domains of life, suggesting a highly conserved and important function of this protein family. The crystal structures of YggS protein family members, including *E. coli* YggS (PDB identifier [ID]: 1W8G), *Saccharomyces cerevisiae* Ybl036c (PDB ID: 1CTS), and *Synechococcus elongatus* PCC 7942 PipY (PDB ID: 5NM8), demonstrated that the proteins bind to PLP via Schiff base linkage with a lysine residue and exhibit structural similarity to the N-terminal

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FIG 1 Metabolic pathway of Met, Thr, Ile, and Val biosynthesis in *E. coli*. Homoserine (Hse) is a branch-point metabolite for the synthesis of Met and Thr. 2-Ketobutyrate, precursor for 2-aminobutyrate or Ile, can be produced from Thr by IlvA. Ile is synthesized from 2-ketobutyrate and pyruvate by the action of IlvBN/IlvIH (AHAS), IlvC, IlvD, and IlvE. Val is produced from pyruvate by same enzymes in the Ile biosynthesis. Abbreviations: CoA, coenzyme A; MetA, homoserine O-succinyltransferase; ThrB, homoserine kinase; ThrC, threonine synthase; IlvA, threonine deaminase; AvtA, valine-pyruvate aminotransferase; GshA, glutamate-cysteine ligase; GshB, glutathione synthase; IlvBN, acetohydroxy acid synthase I; IlvIH, acetohydroxy acid synthase II; IlvC, ketol-acid reductoisomerase; IlvD, dihydroxy-acid dehydratase; IlvE, branched-chain-amino-acid aminotransferase.

domain of bacterial alanine racemase (ALR) and eukaryotic ornithine decarboxylase (ODC), both of which are classified as fold type III PLP enzymes (4, 5). Unlike most other PLP-dependent enzymes, the YggS protein family members exist as monomeric and single-domain proteins, and the *re*-face of PLP is solvent exposed.

Although no biochemical function has yet been identified for this protein family, knockout or mutation of this protein family causes pleiotropic phenotypes in multiple organisms. In *E. coli*, a *yggS* mutation impacts the metabolism of Ile and Val by altering levels of 2-aminobutyrate (2-AB) and/or 2-ketobutyrate (2-KB) (1) (see Fig. 1). The *yggS* mutant of *E. coli* MG1655 accumulates certain metabolites, including 2-AB, 2-KB, γ -glutamyl-2-aminobutyryl-glycine (ophthalmic acid; OA), and Val. The same mutant has decreased coenzyme A content in the log phase and excretes a significant amount of Val in M9 culture medium at the stationary phase during growth at 30°C (1, 2). In contrast, the *yggS* mutant of *E. coli* strain BW25113 does not overproduce Val (2, 3), suggesting that the Val overproduction phenotype is a strain-dependent and/or cultivation condition-dependent phenotype. Nichols et al. showed that *yggS* and *glyA*, encoding serine hydroxymethyl transferase, are a synthetic lethal pair in *E. coli* (6). Impaired growth of the *yggS glyA* double-knockout mutant was also identified in the genome-scale cross of the *glyA* mutant with the entire *E. coli* gene deletion collection (7). Later, it was found that the double mutant can grow in a synthetic medium supplemented with Gly (3), which indicated that the gene pair is a conditional lethal mutant. Together, these data suggest that YggS plays a role(s) in the metabolism of Gly and/or C1 units. Recently, involvement of YggS in PLP homeostasis was reported. Prunetti et al. reported that the *yggS* mutant of *E. coli* BW25113 accumulates PLP

precursor pyridoxine 5'-phosphate (PNP) and is sensitive to excess levels of pyridoxine (PN) (3). Related to this finding, in humans, mutations in PROSC (ortholog of YggS) were identified as the cause of vitamin B₆-dependent epilepsy (8, 9). Analysis of cerebrospinal fluid samples from individuals possessing a PROSC mutation showed low PLP levels and reduced PLP-dependent enzyme activity. Those authors suggested that the YggS protein family is involved in the intracellular homeostasis regulation of PLP, while supplying PLP to apo-PLP enzymes and/or minimizing the toxic effects of PLP aldehyde (3, 8). The involvement of this protein family in the homeostasis of vitamin B₆ and/or amino acids in cyanobacteria was also suggested. In *Synechococcus elongatus*, disruption of the YggS ortholog PipY results in sensitivity to D-cycloserine and β -chloro-D-alanine, antibiotics targeting essential PLP-dependent enzymes. In addition, the *pipY* gene and the putative cysteine synthase *cysK* gene were identified as the synthetic lethal pair (10).

Although mutation of the YggS family protein results in pleiotropic phenotypes, it is likely to have a highly conserved function. The Val overproduction from the *E. coli* *yggS* mutant was complemented by plasmid-borne expression of YggS and orthologs from bacilli (YlmE), yeast (Ybl036cp), and human (PROSC) but not by a YggS mutant lacking PLP-binding ability (1). In addition, the PN sensitivity of the *yggS* mutant was rescued with the YggS orthologs from *Zea mays*, *Arabidopsis thaliana*, *S. cerevisiae*, and human but not with the PROSC mutants identified in patients with vitamin B₆-dependent epilepsy (8). Previous studies had shed light on the importance of YggS protein family in homeostasis of cellular metabolisms; however, little is known about the cause, relevance, and conserved mechanism of these multiple phenotypes.

This study was initiated to probe the PN sensitivity of the *yggS* mutant of *E. coli*. We show here that excess PN induces aberrant PNP accumulation in the *yggS* mutant, which results in the accumulation of Val, which is known to be toxic to *E. coli*. We further showed that a *yggS* mutation perturbs the Thr biosynthetic pathway and results in sensitivity to homoserine (Hse). Our data suggest that the aberrant accumulation of PNP perturbs the Thr biosynthetic pathway and then impacts amino acid homeostasis in the *yggS* mutant. A detailed understanding of each of the phenotypes of *yggS*, such as that reported here, is critical to facilitate studies to determine the molecular function of the YggS protein family.

RESULTS

Exogenous PN induces PNP accumulation and Val accumulation in the *yggS* mutant of *E. coli*. Previous investigation found that an *E. coli* *yggS* mutant was sensitive to excess of pyridoxine (PN) on M9 medium (3), but the molecular mechanism underlying the PN sensitivity phenotype has not been described. The metabolic change that occurred in an isogenic pair of strains upon PN supplementation was examined. We cultivated wild-type (WT) and *yggS* mutant strains of *E. coli* BW25113 expressing green fluorescent protein (GFP) (strains WT_{BW}^{-GFP} and $\Delta yggS_{BW}$ ^{-GFP}, respectively) on M9 plates in the presence or absence of PN (1 mM) at 37°C and determined the levels of intracellular B₆ vitamers and amino acids in the cells. The GFP was expressed to improve the visualization of *E. coli* growth on the plate. A growth assay confirmed that the excess level of PN was toxic to the $\Delta yggS_{BW}$ ^{-GFP} strain (Fig. 2A). B₆ pool analyses showed that the $\Delta yggS_{BW}$ ^{-GFP} mutant accumulated 14 times more PNP in the cells (7.2 ± 1.2 pmol/mg cells) than the WT_{BW}^{-GFP} strain (0.5 ± 0.5 pmol/mg cells) when grown on M9 plates without PN (Table 1). Upon addition of PN, the levels of PNP were significantly elevated in the $\Delta yggS_{BW}$ ^{-GFP} mutant (19 ± 3 pmol/mg cells), reaching 50 times higher than those seen with the WT_{BW}^{-GFP} strain (0.4 ± 0.4 pmol/mg cells). In the *yggS* mutant, PLP and PN pools were not influenced by the addition of PN (Table 1). Notably, exogenous PN did not affect any of the intracellular B₆ vitamers in strain WT_{BW}^{-GFP}. Amino acid analyses found that the intracellular amino acid pools were slightly different between the two strains when grown on M9 plates without PN. The levels of OA, γ -aminobutyric acid (GABA), 2-AB, and Val were ~ 3 times higher in the $\Delta yggS_{BW}$ ^{-GFP} cells than in the WT_{BW}^{-GFP} cells, while the levels of other amino acids

FIG 2 Effect of the excess PN on the growth and intracellular amino acid pool of the *yggS* mutant. (A) The growth of WT_{BW}^{-GFP} and $\Delta yggS_{BW}^{-GFP}$ strains on M9 plates containing 0.1% L-arabinose at 37°C. Filter discs containing distilled water (N.C.) or 1 mM PN (PN) were placed on the plates. Exogenous PN was toxic to strain $\Delta yggS_{BW}^{-GFP}$ but not to strain WT_{BW}^{-GFP} . (B and C) Representative HPLC chromatograms of the amino acid analyses. (B) In the absence of PN, the amino acid pools of WT_{BW}^{-GFP} and $\Delta yggS_{BW}^{-GFP}$ cells were slightly different. (C) Extracellular PN significantly stimulated the production of ophthalmic acid (OA), γ -aminobutyric acid (GABA), 2-aminobutyrate (2-AB), and Val in the $\Delta yggS_{BW}^{-GFP}$ strain.

were not significantly different (Fig. 2B). Interestingly, when the $\Delta yggS_{BW}^{-GFP}$ strain was grown with PN, the amino acid pools were significantly altered. The $\Delta yggS_{BW}^{-GFP}$ strain accumulated substantial amounts of OA, GABA, 2-AB, and Val, whose concentrations were 10, 7, 40, and 10 times higher than those accumulated by the WT_{BW}^{-GFP} strain, respectively (Fig. 2C). These data showed that in the absence of *yggS*, the presence of exogenous PN significantly increased the intracellular concentrations of PNP, GABA, 2-AB, and Val in *E. coli*.

Excess PN induces PNP and causes Val toxicity in the *yggS* mutant. The high concentration of Val in the *yggS* mutant grown with the excess of PN suggested an explanation for the resulting growth inhibition. The *E. coli* K-12 strain is sensitive to Val

TABLE 1 Effect of excess PN on intracellular B_6 pool of the *yggS* mutant^a

Strain	pmol of indicated B_6 vitamer/mg cells $\pm$ SD					
	PLP		PNP		PMP	
	No PN	PN	No PN	PN	No PN	PN
<i>E. coli</i> WT_{BW}^{-GFP}	51 $\pm$ 3	71 $\pm$ 5	0.5 $\pm$ 0.5	0.4 $\pm$ 0.4	76 $\pm$ 2	111 $\pm$ 6
<i>E. coli</i> $\Delta yggS_{BW}^{-GFP}$	48 $\pm$ 2	65 $\pm$ 4	7.2 $\pm$ 1.2	19 $\pm$ 3	188 $\pm$ 10	209 $\pm$ 7
<i>E. coli</i> $\Delta thrB_{BW}^{-GFP}$	32 $\pm$ 7	40 $\pm$ 3	0.3 $\pm$ 0.2	0.3 $\pm$ 0.3	56 $\pm$ 7	89 $\pm$ 7
<i>E. coli</i> $\Delta thrB \Delta yggS_{BW}^{-GFP}$	31 $\pm$ 2	42 $\pm$ 6	3.9 $\pm$ 0.5	15 $\pm$ 3	64 $\pm$ 3	99 $\pm$ 14
<i>E. coli</i> $\Delta thrC_{BW}^{-GFP}$	24 $\pm$ 4	32 $\pm$ 3	0.5 $\pm$ 0.01	1.1 $\pm$ 0.3	50 $\pm$ 5	76 $\pm$ 7
<i>E. coli</i> $\Delta thrC \Delta yggS_{BW}^{-GFP}$	23 $\pm$ 1	30 $\pm$ 4	3.5 $\pm$ 0.3	13 $\pm$ 2.4	56 $\pm$ 3	86 $\pm$ 13

^a*E. coli* strains were grown on the M9 plate containing 0.1% L-arabinose at 37°C for 16 h in the absence or presence of excess PN (1 mM). Note that the $\Delta thrB_{BW}^{-GFP}$ and $\Delta thrB \Delta yggS_{BW}^{-GFP}$ strains were grown in the presence of 0.4 mM Thr. The $\Delta thrC_{BW}^{-GFP}$ and $\Delta thrC \Delta yggS_{BW}^{-GFP}$ strains were grown with 0.4 mM Thr. The cells were collected by scraping, and the total intracellular B_6 vitamers were quantified as described in Materials and Methods. The *yggS* mutants accumulated PNP, and exogenous PN further elevated the PNP level in the *yggS* mutant. Data represent means $\pm$ standard deviations of results from at least three independent experiments.

FIG 3 Effect of overexpression of PdxH or AHASII on the PN sensitivity or Val productivity of the *yggS* mutant. (A) Strain WT_{BW}^{-pBAD}, ΔyggS_{BW}^{-pBAD}, ΔyggS_{BW}^{-pdxH}, or ΔyggS_{BW}^{-AHASII} was grown on M9 plates at 37°C. Filter discs containing distilled water (N.C.) or 1 mM PN (PN) were placed on the plates. The expression of either of AHASII or PdxH in the *yggS* mutant eliminated the PN sensitivity. (B and C) Effect of *pdxH* expression on the PNP levels (B) and Val concentrations (C) in the *yggS* mutant. The ΔyggS_{BW}^{-pBAD} and ΔyggS_{BW}^{-pdxH} strains were cultivated on the M9 plate in the presence of PN at 37°C. Intracellular concentrations of PNP and Val were quantified as described in Materials and Methods. The expression of PdxH significantly decreased the PNP and Val levels in the *yggS* mutant. Error bars show standard deviations of results from at least three independent experiments.

(11–13). Excess Val inhibits the two acetohydroxy acid synthases (AHASs) active in *E. coli* K-12 strain, which are encoded by *ilvBN* (AHAS I) and *ilvIH* (AHAS III). Acetohydroxy acid synthase activity is required for branched-chain amino acid biosynthesis, and inhibition of such activity can lead to the accumulation of the toxic metabolite 2-ketobutyrate (2-KB) (11–13). When a Val-insensitive AHAS (encoded by *ilvGM*) from *Salmonella enterica* was expressed in the *yggS* mutant (strain ΔyggS_{BW}^{-AHASII}), growth on the M9 plate in the presence of PN was restored (Fig. 3A). These data allowed the conclusion that the toxicity of PN to a *yggS* mutant was due to the increased level of Val.

We considered whether the increased PNP accumulation in a *yggS* mutant could be responsible for the Val overproduction. Consistently, it was reported previously that an *E. coli* strain lacking pyridoxine/pyridoxamine 5'-phosphate oxidase (PdxH) excretes Glu and unknown metabolites that trigger Val inhibition (14). PdxH catalyzes the oxidation of PNP (and pyridoxamine phosphate [PMP]) to PLP in the final step of vitamin B₆ biosynthesis (15), and therefore the *pdxH* mutant might accumulate PNP and PMP. We expressed *pdxH* in the *yggS* mutant (strain ΔyggS_{BW}^{-pdxH}) in an effort to lower the intracellular PNP concentration and examined the effect on the PN sensitivity and thus Val accumulation. As shown in Fig. 3A, the expression of *pdxH* eliminated the inhibitory effect of PN on the *yggS* mutant. As expected, B₆ pool analysis showed that expression of *pdxH* significantly decreased the intracellular PNP levels in the *yggS* mutant (Fig. 3B). Significantly, expression of *pdxH* in the *yggS* mutant resulted in a reduction in the intracellular concentration of Val to the level present in the wild-type strain (Fig. 3C). These data support the conclusion that elevation of the level of intracellular PNP in a *yggS* mutant causes an increase in Val by an uncharacterized mechanism. The increased Val prevents growth by inhibition of AHAS and the resulting accumulation of 2-KB.

Homoserine (Hse) metabolism is altered in a *yggS* mutant. A *yggS* mutation was shown previously to affect the 2-AB/2-KB metabolic pathway(s) and to result in overproduction of Val in the *E. coli* cells (1, 2). Those and other observations prompted us to query growth of the *yggS* mutant in response to several amino acids or keto acids involving in the branched-chain amino acid biosynthesis pathway and related pathways (Thr, 2-AB, 2-KB, L-homoserine [Hse], or Met). The wild-type strain (WT_{BW}^{-pU0}) and the *yggS*-deficient strain (mutant ΔyggS_{BW}^{-pU0}) had indistinguishable growth rates in

FIG 4 Inhibition of the *yggS* mutant by Hse and its alleviation by *thrC* overexpression. (A to D) Growth curves of the WT_{BW}^{-pU0}, $\Delta yggS_{BW}$ ^{-pU0}, and $\Delta yggS_{BW}$ ^{-yggS} strains in liquid M9 medium without additive (A) or with 1 mM Hse (B), 0.1 mM Met and 1 mM Hse (C), or 0.1 mM Ile and 1 mM Hse (D) at 30°C are represented. (E) Growth of strains WT_{BW}^{-pCA24N}, $\Delta yggS_{BW}$ ^{-pCA24N}, and $\Delta yggS_{BW}$ ^{-thrC} in M9 medium containing 1 mM Hse at 30°. (F) Comparison of the growth levels of strains WT_{BW}^{-pBAD24}, $\Delta yggS_{BW}$ ^{-pBAD24}, and $\Delta yggS_{BW}$ ^{-AHASII} in M9 medium containing 1 mM Hse at 30°C. Error bars show standard deviations of results from at least three independent experiments.

M9 medium (Fig. 4A) and in the presence of Thr, 2-AB, 2-KB, or Met (data not shown). In contrast, when 1 mM Hse was added to the M9 medium at 30°C, the $\Delta yggS_{BW}$ ^{-pU0} mutant exhibited a nearly 10-h lag before achieving a growth rate similar to that of the WT_{BW}^{-pU0} strain (Fig. 4B). The duration of the lag was prolonged with an increased Hse concentration in the medium. Plasmid-borne expression of the *yggS* strain completely eliminated the growth defect caused by Hse, confirming that the phenotype was due to the *yggS* deletion (Fig. 4B, diamond). Further growth assays showed that the Hse sensitivity of the $\Delta yggS_{BW}$ ^{-pU0} mutant was overcome in the presence of exogenous Ile but not in the presence of Met (Fig. 4C and D). Supplementation with 1 mM Thr or 1 mM 2-AB, each of which can be converted to Ile *in vivo*, also effectively eliminated the Hse sensitivity (data not shown). Surprisingly, when grown at 37°C, the WT_{BW}^{-pU0} strain

FIG 5 Effect of Hse with respect to the metabolites of the Thr, Ile, and Val biosynthetic pathways of the *yggS* mutant. The intracellular concentrations of amino acids and keto acids of the WT_{BW}^{-pU0} and ΔyggS_{BW}^{-pU0} strains grown in M9 medium supplemented with 1 mM Hse at 30°C are shown in panels A and C, respectively. HPLC chromatograms of the amino acid analyses showing P-Hse accumulation in the ΔyggS_{BW}^{-pU0} strain are shown in panel B. Error bars show standard deviations of results from at least three independent experiments.

and the ΔyggS_{BW}^{-pU0} mutant were inhibited by exogenous Hse to the same extent (data not shown). These data suggested that the effect of YggS with respect to its role in Hse metabolism is affected by temperature and thus is likely to be an indirect effect. We sought to investigate this phenotype further to better understand the molecular function of YggS.

Exogenous Hse induces accumulation of metabolites in the Thr/Ile/Val biosynthetic pathway in the *yggS* mutant. Intracellular concentrations of amino and keto acids were analyzed in the WT_{BW}^{-pU0} strain and in the ΔyggS_{BW}^{-pU0} mutant in the presence and absence of Hse. In the absence of Hse, the intracellular amino acid composition of the WT_{BW}^{-pU0} strain was indistinguishable from that of the ΔyggS_{BW}^{-pU0} mutant, with the exception of ophthalmic acid (OA), which accumulated to levels approximately 10 times higher in the ΔyggS_{BW}^{-pU0} mutant than in the WT_{BW} strain (2). In the presence of Hse, the ΔyggS_{BW}^{-pU0} strain accumulated a significantly more Thr, Val, and Ile in the cells than the wild-type strain, with 5-fold-, 2-fold-, and 8-fold-higher concentrations, respectively (Fig. 5A). In addition, the ΔyggS_{BW}^{-pU0} strain accumulated a significant amount of L-O-phosphohomoserine (P-Hse) in the cells (Fig. 5B). A significant concentration (~50 μM) of P-Hse was also detected in the stationary-phase medium with the ΔyggS_{BW}^{-pU0} strain but not in the cells or in the medium with the WT_{BW}^{-pU0} strain. In contrast, the levels of 2-AB and Met were slightly lower in the ΔyggS_{BW}^{-pU0} strain than in the WT_{BW}^{-pU0} strain (Fig. 5A). The levels of other proteino-genic amino acids were not significantly influenced by the *yggS* mutation. Results of the keto acid analysis are shown in Fig. 5C. Consistent with the results of the amino acid analyses, when grown in M9 medium supplemented with Hse, the ΔyggS_{BW}^{-pU0} cells accumulated more α-ketoisovalerate (KIV; precursor of Val), α-ketoisocaproate (KIC;

precursor of Ile), α -keto- β -methylvalerate (KMV; precursor of Leu), and α -ketoglutarate than the WT_{BW}^{-pUO} cells. The results suggest there is increased flux through the biosynthetic pathways for branched-chain amino acids in the *yggS* mutant.

The accumulation of P-Hse in the *yggS* mutant raised the formal possibility that P-Hse was a substrate for YggS. Many PLP-dependent enzymes exhibit a shift of the peak at around 420 nm derived from Schiff base linkage between PLP and the Lys residue of the protein upon substrate binding. Addition of P-Hse to purified YggS did not induce any spectrum changes (Fig. S1). Further, incubation of YggS and P-Hse in the presence or absence of keto acid (pyruvate or α -ketoglutarate) did not cause any changes in P-Hse concentrations in the reaction mixture that would suggest that a catalytic reaction had taken place. These experiments failed to detect any reactivity of YggS with P-Hse, although as negative results they are not definitive. The P-Hse accumulation could suggest that threonine synthase (ThrC) activity is the rate-limiting step in the catabolism of Hse in the *yggS* mutant. The ThrC activity in the $\Delta yggS_{BW}$ strain (5.1 ± 0.2 nmol/min/mg protein) was ~ 1.3 times higher than that in the WT_{BW} strain (4.0 ± 0.1 nmol/min/mg protein). These data did not support the idea that the P-Hse accumulation was due to low activity of ThrC in the *yggS* mutant.

Sensitivity to Hse correlates with P-Hse accumulation. Hse is the branch point intermediate in the pathway for Thr and Met biosynthesis in *E. coli* (Fig. 1). Homoserine O-succinyltransferase (encoded by *metA*) and homoserine kinase (encoded by *thrB*) catalyze the initial step for Met and Thr biosynthesis from Hse (16, 17). Deletion of *metA* or *thrB* eliminates the metabolic flux into Met or Thr from Hse. A *yggS*-deficient strain was constructed in both a *metA* background and a *thrB* background, and the strains were assessed for growth in the presence or absence of Hse when the required supplements (Met and Thr, respectively) were provided. In the absence of Hse, the $\Delta metA \Delta yggS_{BW}^{-pUO}$ strains and $\Delta thrB \Delta yggS_{BW}^{-pUO}$ strains showed growth indistinguishable from that of the parental control strains (mutants $\Delta metA_{BW}^{-pUO}$ and $\Delta thrB_{BW}^{-pUO}$) (Fig. 6A and D). In the presence of Hse, the *thrB* mutation, but not the *metA* mutation, suppressed the Hse sensitivity of the *yggS* mutant (Fig. 6B and E). These data resulted in the conclusion that the flux through the Thr biosynthetic pathway was required to generate Hse sensitivity of an *yggS* mutant of *E. coli*. It is formally possible that P-Hse negatively impacts the growth of *E. coli*. To test this possibility, we overexpressed ThrC, catalyzing conversion of P-Hse to Thr in the *yggS* mutant, which is expected to decrease the intracellular concentration of P-Hse. The *yggS* mutant harboring the plasmid carrying *thrC* was not sensitive to Hse (Fig. 4E). These data are consistent with the accumulation of P-Hse being the primary cause of the Hse sensitivity of the *yggS* mutant. Because the *yggS* mutant grown with Hse also accumulated Val and KIV in the cells, it was formally possible that the Hse sensitivity was indirectly due to Val toxicity. This possibility was eliminated as a consequence of the fact that expression of Val-insensitive AHAS (AHASII; *ilvGM*) in the *yggS* mutant failed to eliminate the growth defect caused by Hse (Fig. 4F).

Hse perturbs amino acid metabolism in the *yggS* mutant via PNP accumulation. In the absence of Hse, a $\Delta yggS_{BW}^{-pUO}$ strain accumulated a 10-times-higher concentration of PNP than was seen with the WT_{BW}^{-pUO} strain (3.3 ± 0.2 and 0.3 ± 0.3 pmol/mg cells, respectively), while the other B_6 vitamers were similar in the two strains (Table 2). When Hse was provided in the medium, the level of PNP content was further elevated in the *yggS* mutant but not in the wild-type strain. In the presence of Hse, the $\Delta yggS_{BW}^{-pUO}$ mutant accumulated PNP at 8.0 ± 0.2 pmol/mg cells, which was a level >80 times higher than that seen with the WT_{BW} strain and 2.4 times higher than that seen with the $\Delta yggS_{BW}^{-pUO}$ mutant grown in the absence of Hse (Table 2). In contrast, the levels of PLP were not significantly different between the two strains.

The elevation of PNP levels in the *yggS* mutant suggested that, like the PN sensitivity, increased PNP levels represent a primary cause of Hse sensitivity. Growth was conducted with the $\Delta yggS_{BW}^{-PdxH}$ strain, which overexpresses PdxH. The expression of *pdxH* did not eliminate the Hse sensitivity of the *yggS* mutant (Fig. 7A), despite the finding that there was a significant decrease in the level of PNP in the $\Delta yggS_{BW}^{-PdxH}$

FIG 6 Effect of *yggS* mutation in the *metA* or *thrB* background on the growth or amino acid pools of the strains examined. (A and B) The $\Delta metA_{BW-pU0}$ (●) and $\Delta metA \Delta yggS_{BW-pU0}$ (△) strains were grown in M9 medium supplemented with 0.1 mM Met in the absence (A) or presence (B) of 1 mM Hse. (C) Intracellular amino acid concentrations of the $\Delta metA_{BW-pU0}$ and $\Delta metA \Delta yggS_{BW-pU0}$ strains grown in the presence of 0.1 mM Met and 1 mM Hse. (D and E) The growth of the $\Delta thrB_{BW-pU0}$ (●) and $\Delta thrB \Delta yggS_{BW-pU0}$ (△) strains in the M9 medium supplemented with 0.5 mM Thr (D) or with 0.5 mM Thr and 1 mM Hse (E). (F) Intracellular amino acid concentrations of the $\Delta thrB_{BW-pU0}$ and $\Delta thrB \Delta yggS_{BW-pU0}$ strains grown in M9 medium supplemented with 0.5 mM Thr and 1 mM Hse. The *yggS thrB* double mutant did not show Hse sensitivity or perturbation of the amino acid pool. Data are presented as means $\pm$ standard deviations of results from at least three independent experiments.

mutant (Fig. 7B). Further increases in the expression of *pdxH*, aiming at complete elimination of PNP (by increasing the concentration of arabinose to up to 0.2%), instead inhibited the growth of both the wild-type strain and the *yggS* mutant. Expression of *pdxH* in the *yggS* mutant significantly reduced the intracellular concentrations of P-Hse and Val (Fig. 7C and D). There is no obvious explanation for the slow growth of the $\Delta yggS_{BW-PdxH}$ mutant in the presence of Hse at present. In total, our data showed that PNP accumulation correlates with the P-Hse (and Val) overproduction found in the *yggS* mutant but neither metabolite was directly linked to the growth defect that is caused by the presence of Hse.

Flux through the Thr biosynthetic pathway is required for the amino acid pool perturbation but for not PNP accumulation in the *yggS* mutant. We found that the $\Delta thrB_{pU0}$ and $\Delta thrB \Delta yggS_{BW-pU0}$ mutants grown in the presence of Hse exhibited almost identical intracellular amino acid compositions, including Thr, Ile, and Val

TABLE 2 Effect of exogenous Hse on intracellular B₆ pool of the *yggS* mutant^a

Strain	pmol of indicated B ₆ vitamer/mg cells $\pm$ SD					
	PLP		PNP		PMP	
	No Hse	Hse	No Hse	Hse	No Hse	Hse
<i>E. coli</i> WT _{BW-pU0}	14 $\pm$ 2	15 $\pm$ 1	0.3 $\pm$ 0.3	ND	24 $\pm$ 4	26 $\pm$ 3
<i>E. coli</i> $\Delta yggS_{BW-pU0}$	9.0 $\pm$ 2	17 $\pm$ 2	3.3 $\pm$ 0.2	8.0 $\pm$ 0.2	19 $\pm$ 2	29 $\pm$ 3

^aStrains WT_{BW-pU0} and $\Delta yggS_{BW-pU0}$ were grown in the M9 medium in the presence or absence of 1 mM Hse at 30°C. Log-phase cells were collected, and total intracellular B₆ vitamers were quantified as described in Materials and Methods. The *yggS* mutants accumulated PNP, and exogenous Hse further elevated the PNP level in the *yggS* mutant. Data represent means $\pm$ standard deviations of results from at least three independent experiments. ND, not detected.

FIG 7 Effect of *pdxH* expression on Hse sensitivity. (A) Growth of strains WT_{BW}-pBAD, Δ*yggS*_{BW}-pBAD, and Δ*yggS*_{BW}-pdxH in the M9 medium supplemented with 1 mM Hse at 30°C. (B) Intracellular concentrations of PNP in the three strains grown in the M9 medium supplemented with 1 mM Hse. ND, not detected. (C) HPLC chromatograms representative of the amino acid analyses, showing a decreased P-Hse concentration in strain Δ*yggS*_{BW}-pdxH. (D) Intracellular concentrations of Val in the three strains grown in the M9 medium supplemented with 1 mM Hse. Error bars show means $\pm$ standard deviations of results from at least three independent experiments.

concentrations (Fig. 6E). This result suggests that the Thr biosynthetic pathway flux is involved in the perturbation of the intracellular amino acid pools in the *yggS* mutant. Importantly, the same was true in the *yggS* mutant grown with excess PN on the M9 plate, since exogenous PN did not compromise the growth of the Δ*thrB* Δ*yggS*_{BW}-GFP mutant (Fig. 8A). No further alteration of the intracellular amino acid pool that included OA, GABA, 2-AB, and Val was detected in the Δ*thrB* Δ*yggS*_{BW}-GFP mutant by PN (Fig. 8B and C). Furthermore, we found that the exogenous PN did not compromise the growth of the Δ*thrC* Δ*yggS*_{BW}-GFP mutant. No amino acid pool alteration was induced in the Δ*thrC* Δ*yggS*_{BW}-GFP mutants by the excess of PN (Fig. S2). These observations indicated that the alteration of the Thr biosynthetic pathway affected the disturbance of the intracellular amino acid pools in the *yggS* mutant.

Excess 2-AB/2-KB induces Val overproduction in *E. coli* cells (1). Therefore, Val overproduction can be explained by the increasing supply of 2-AB/2-KB mediated by the increased production of the precursor Thr. In contrast, the P-Hse accumulation can be explained by the relative increase or decrease of ThrB or ThrC enzyme activity in the *yggS* mutant. We first examined whether PNP directly influences the two enzymes. However, our *in vitro* experiments failed to detect any activation or inhibition effects of PNP (at up to 100 μ M PNP) on purified ThrB or ThrC enzymes under the conditions examined. In contrast, we found that the levels of ThrC in the cell extract of *yggS*

(A)

(B)

(C)

FIG 8 Effect of ThrB mutation with respect to PN sensitivity and amino acid pool disturbance in the *yggS* mutant. (A) The growth of the *thrB*_{BW}-GFP and *ΔthrB ΔyggS*_{BW}-GFP strains on M9 plates containing 0.4 mM Thr and 0.1% L-arabinose. Filter discs containing 20 μ l of distilled water (N.C.) or 1 mM pyridoxine (PN) were placed on the plates. The *ΔthrB ΔyggS*_{BW}-GFP strain did not show PN sensitivity. (B and C) HPLC chromatograms representing the amino acid analyses performed in the absence (B) or presence (C) of PN. Excess PN did not induce perturbation of the amino acid pool in the *yggS* mutant.

mutant (23.4 ± 0.2 nmol/min/mg protein) were 2 times higher than those seen with the wild-type strain (11.4 ± 0.4 nmol/min/mg protein) under conditions of growth in the M9 medium.

Importantly, B₆ pool analyses revealed that mutation of *thrB* or *thrC* does not influence the extent of the PNP accumulation in the *yggS* mutant (Table 1). In the absence of PN, the *ΔthrB ΔyggS*_{BW}-GFP mutant accumulated PNP at 3.9 ± 0.5 pmol/mg cells, which was 15 times higher than the level seen with the *ΔthrB*_{BW}-GFP mutant (0.3 ± 0.2 pmol/mg cells). When PN was provided, *ΔthrB ΔyggS*_{BW}-GFP cells accumulated considerably more PNP (15 ± 3 pmol/mg cells), and the level was 45 times higher than the accumulation in the parental strain (Table 1) and comparable to that observed with the *ΔyggS*_{BW}-GFP cells. Similar results were obtained with the *thrC* mutants (Table 1). Our data thus suggest that the perturbation of the Thr biosynthetic pathway flux plays a minor role in regulating PNP levels in the *yggS* mutant.

DISCUSSION

Previous studies showed that the disruption of *yggS* in *E. coli* causes pleiotropic phenotypes, especially with respect to related to amino acid and vitamin B₆ metabolisms. However, little had been known about the cause, mechanism, and relevance of the phenotypes for the *yggS* mutant. The current study showed that a high concentration of intracellular PNP is the root cause for the two apparently unrelated phenotypes previously reported in a *yggS* mutant, the PN sensitivity and Val overproduction. In the absence of *yggS*, the *E. coli* cell accumulates PNP, which is further induced by certain stresses, including those represented by excess PN or Hse levels. Elevated levels of PNP itself do not prevent *E. coli* growth but influence flux through Thr biosynthesis and then perturb the Ile/Val biosynthetic pathway to accumulate some metabolite in the pathway under certain conditions. Accumulation of Val is likely to prevent growth of the *yggS* mutant.

The mechanism of the PNP-dependent perturbation of the Thr biosynthetic pathway

FIG 9 Biosynthetic pathway of PNP to the coenzyme PLP and the salvage pathway of PLP in *E. coli*. PNP is synthesized by the deoxyxylulose 5-phosphate (DXP)-dependent pathway. It is subsequently oxidized by PNP/PMP oxidase (PdxH) to produce PLP. PLP is recycled through a salvage pathway involving pyridoxal kinase (PdxK and PdxY), pyridoxal phosphatase (YbhA), and PdxH. The putative reactions in this pathway are represented by broken arrows.

is YggS independent, and its nature is currently unclear. We observed increasing ThrC activity in the cell extract of *yggS* mutant, which suggests increasing expression of the *thr* operon consisting of the *thrL*, *thrA*, *thrB*, and *thrC* genes (18). The *thr* operon is controlled by levels of certain amino acids, including Thr and Ile (19). PLP mostly exists in a protein-bound form, while free-form PNP may exist in the cells. The concentration of PNP in the *yggS* mutant was equal to or greater than that of PLP, as judged by our B₆ pool analyses (Table 1; see also Table 2). Therefore, it is possible that PNP inhibits some PLP-dependent enzymes and/or transcriptional regulators and then alters the amino acid pool and expression of the *thr* operon. Such an indirect mode of action could explain the cultivation condition-dependent and/or strain-dependent pleiotropic phenotypes of the *yggS* mutant.

Our next challenge will be identification of the cause and mechanism of the PNP accumulation. We found that the *yggS* mutant accumulated PNP in all genetic backgrounds (*thrB*, *thrC*, *metA*, and *E. coli* MG1655) and under all cultivation conditions (LB and M9 medium containing Casamino Acids at both 30°C and 37°C) examined, indicating the essential role of YggS in low-level maintenance of PNP. A previous study (3) and our *in vitro* investigation failed to detect any *in vitro* reactivity of YggS toward PNP, which suggested indirect involvement in the homeostasis of PNP. In *E. coli*, PNP is synthesized by the deoxyxylulose 5-phosphate (DXP)-dependent pathway through the action of 4-hydroxythreonine-4-phosphate dehydrogenase (PdxA) and PNP synthase (PdxJ) (20–24). It is subsequently oxidized by PdxH to produce PLP (15). Once PLP is produced, it is recycled through a salvage pathway involving pyridoxal kinase (PdxK and PdxY) (25, 26), pyridoxal phosphatase (YbhA) (27), and PNP/PMP oxidase (PdxH) (Fig. 9). Either or both of increasing supply or decreasing metabolism of PNP can explain the PNP accumulation in the *yggS* mutant. One plausible model for the PNP accumulation is involvement of YggS in the maintenance of PdxH activity *in vivo*. Elevation of PMP levels in addition to PNP in the *yggS* mutant (Table 2) could support

this hypothesis. Interestingly, the distribution of the YggS protein family is different from that of PdxH; many organisms that utilize a DXP-independent pathway to synthesize PLP lack PdxH. YggS orthologs, including those of human and yeast exhibiting 20% to 40% sequence identity, are capable of complementation of the phenotypes of *yggS* mutant (1, 3). These facts suggest that YggS is indirectly involved in the maintenance of PdxH activity. It is possible that the YggS protein family is involved in the low-level maintenance of free PLP by storing PLP in the protein. The YggS protein family is abundant in eukaryotic cells. In *S. cerevisiae*, the copy numbers for 90% of the yeast proteins are within the vicinity of 2,000, while there are 17,500 copies of YBL036c in one cell. In HeLa cells, the median level of protein expression is 21,000 copies per cell, but approximately 200,000 copies of PROSC protein are expressed in a cell (28). Therefore, the disruption of this protein family could increase the level of the free PLP pool in the cells. The elevation of cellular PLP levels can increase the levels of holo-PLP-dependent enzymes, as well as those of the unwanted PLP adducts formed with proteins and small metabolites. The elevation of free PLP levels could cause feedback inhibition of PdxH and the accumulation of PNP in the cells (15). Further studies will be required to examine these hypotheses and to identify the molecular function of YggS protein family. We think that the data presented here provide an important starting point for further study of this protein family.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. The bacterial strains used in this study were generated from *E. coli* BW25113 (Table 3). M9-glucose and LB media were prepared as described previously (1). The *E. coli* wild-type strain, i.e., strain BW25113, and its derivatives (Keio collection) and pCA24N and the derivatives (ASKA collection) were obtained from the National Bioresource Project (NBRP; Keio collection) (29, 30). Antibiotics were added as needed to the M9 and LB media at the following concentrations: ampicillin, 100 μ g/ml; kanamycin, 30 μ g/ml; chloramphenicol, 30 μ g/ml. L-Arabinose and isopropyl- β -D-thiogalactoside (IPTG) were added to the medium to reach final concentrations of 0.1 mM and 0.02%, respectively. Amino acids were purchased from Wako Pure Chemical Corporation (Osaka, Japan) or Sigma-Aldrich (St. Louis, MO, USA). PLP, PL, and PN were purchased from Nacalai Tesque (Kyoto, Japan). PNP was prepared by the reduction of PLP with NaBH₄ in aqueous solution.

Growth analyses. *E. coli* strains were precultured overnight in LB at 30°C. The cells (200 μ l) were washed twice with 1 ml PBS (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 140 mM NaCl, 2.7 mM KCl, pH 7.5) and resuspended in 1 ml of PBS. For the liquid culturing, the cells were inoculated into M9 medium at an initial optical density at 600 nm (OD₆₀₀) of 0.01 to 0.05. The cells were grown to full density at 30°C with shaking. Amino acids were added at the indicated concentrations. Cells growth was recorded using an OD-Monitor C&T apparatus (Taitec Co., Ltd., Koshigaya, Japan) and glass test tubes (16.5 mm in diameter by 165 mm in height) or an ELx808 absorbance microplate reader (BioTek, Winooski, VT, USA) and 96-well plates. For the growth on the M9 plate, the *E. coli* cells in the PBS were further diluted 100-fold and spread (200 μ l) onto an M9 plate. After gentle drying, a paper disc (6 mm in diameter) was placed on the plate, and 20 μ l of 1 mM PN was spotted on the disc. The plate was incubated at 37°C and was photographed with an LuminoGraph II imaging system (ATTO Corp., Tokyo, Japan).

Genetic techniques. Deletion of the *yggS* genes was performed using the bacteriophage λ -Red recombinase system described previously by Datsenko and Wanner (31). The Δ *metA* Δ *yggS*_{BW} strain was constructed as follows. The *E. coli* JW3973-KC strain possessing pCP20 was streaked on an LB plate and grown at 42°C, forming strain JW3973-KC^{kan-}. A PCR product was generated with primers *yggS*-H1 and *yggS*-H2 (32) using Tks Gflex DNA polymerase (TaKaRa) and pKD13 as a template. The PCR product was purified from agarose gel and electroporated into the JW3973-KC^{kan-} strain harboring pKD46. The resultant transformants that appeared on an LB plate containing 30 μ g/ml kanamycin were screened by PCR for the appropriate insertion of the kanamycin resistance gene with primers *yggS*-200up and *yggS*-300down (32). Construction of the Δ *thrB* Δ *yggS*_{BW} mutant or the Δ *thrC* Δ *yggS*_{BW} mutant was performed in a similar way using JW0002-KC or JW0003-KC as the parental strain.

Construction of pBAD-PdxH was performed as follows. The *E. coli* *pdxH* gene was amplified by PCR with KOD-plus2 DNA polymerase (Toyobo). The primers used were *pdxH*-pBAD-fw (GGCCGCCATGGCAATGCTCTGATAACGACGAATTGCAGCAAA) and *pdxH*-pBAD-rv (GGCCGGAATTCTCAGGTGCAAGACGATCAATCTTCCA). The resultant PCR product was purified by agarose gel electrophoresis, digested by NcoI and EcoRI (NEB Japan), and ligated into pBAD-Myc/HisC vector (Invitrogen) digested with the same restriction enzymes. Constructs were transformed into *E. coli* XL10-Gold (Stratagene). The sequence of the *pdxH* insertion was confirmed by sequencing. Expression of active PdxH was confirmed by complementation of a *pdxH*-deficient *E. coli* strain.

Amino acid analysis. Cells were grown in the M9 medium at 30°C to a final OD₆₀₀ of 0.5 or on M9 plates at 37°C for 24 h. The cell pellet was washed once with ice-cold PBS and resuspended in 5% (wt/vol) trichloroacetic acid (TCA) solution (100 μ l for 10 mg wet cells) as described previously (33). After vortex mixing and incubation at 4°C for 30 min, cell debris was removed by centrifugation at 20,000 $\times$ g for 20 min at 4°C. Amino acids were derivatized as described previously (33). Briefly, 25 μ l of the diluted

TABLE 3 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
<i>E. coli</i> WT _{BW}	<i>E. coli</i> K-12 BW25113 wild type; $\Delta(\text{araD-araB})567 \Delta\text{lacZ4787}(\text{::rrnB-3})$	Keio collection
<i>E. coli</i> $\Delta\text{yggS}_{\text{BW}}$	<i>rph-1 \Delta(rhaD-rhaB)568 hsdR514</i>	Keio collection
<i>E. coli</i> $\Delta\text{metA}_{\text{BW}}$	BW25113 $\Delta\text{yggS}::\text{kan}$ (JW2918-KC)	Keio collection
<i>E. coli</i> $\Delta\text{thrB}_{\text{BW}}$	BW25113 $\Delta\text{metA}::\text{kan}$ (JW3973-KC)	Keio collection
<i>E. coli</i> $\Delta\text{thrC}_{\text{BW}}$	BW25113 $\Delta\text{thrB}::\text{kan}$ (JW0002-KC)	Keio collection
<i>E. coli</i> JW3973-KC ^{kan-}	BW25113 $\Delta\text{thrC}::\text{kan}$ (JW0003-KC)	Keio collection
<i>E. coli</i> JW0002-KC ^{kan-}	BW25113 ΔmetA	This study
<i>E. coli</i> JW0003-KC ^{kan-}	BW25113 ΔthrB	This study
<i>E. coli</i> $\Delta\text{metA} \Delta\text{yggS}_{\text{BW}}$	BW25113 ΔthrC	This study
<i>E. coli</i> $\Delta\text{thrB} \Delta\text{yggS}_{\text{BW}}$	BW25113 $\Delta\text{metA} \Delta\text{yggS}::\text{kan}$	This study
<i>E. coli</i> $\Delta\text{thrC} \Delta\text{yggS}_{\text{BW}}$	BW25113 $\Delta\text{thrB} \Delta\text{yggS}::\text{kan}$	This study
<i>E. coli</i> WT _{BW} ^{-pu0}	BW25113 $\Delta\text{thrC} \Delta\text{yggS}::\text{kan}$	This study
<i>E. coli</i> WT _{BW} ^{-GFP}	WT _{BW} /pU0	1
<i>E. coli</i> WT _{BW} ^{-pBAD24}	WT _{BW} /pBAD18-GFP	This study
<i>E. coli</i> WT _{BW} ^{-pBAD}	WT _{BW} /pBAD24	This study
<i>E. coli</i> WT _{BW} ^{-pCA24N}	WT _{BW} /pBAD-Myc-HisA	This study
<i>E. coli</i> $\Delta\text{yggS}_{\text{BW}}$ ^{-pu0}	WT _{BW} /pCA24N	This study
<i>E. coli</i> $\Delta\text{yggS}_{\text{BW}}$ ^{-yggS}	$\Delta\text{yggS}_{\text{BW}}$ /pU0	1
<i>E. coli</i> $\Delta\text{yggS}_{\text{BW}}$ ^{-GFP}	$\Delta\text{yggS}_{\text{BW}}$ /pUS	1
<i>E. coli</i> $\Delta\text{yggS}_{\text{BW}}$ ^{-pBAD24}	$\Delta\text{yggS}_{\text{BW}}$ /pBAD-GFP	This study
<i>E. coli</i> $\Delta\text{yggS}_{\text{BW}}$ ^{-pBAD}	$\Delta\text{yggS}_{\text{BW}}$ /pBAD24	This study
<i>E. coli</i> $\Delta\text{yggS}_{\text{BW}}$ ^{-AHASII}	$\Delta\text{yggS}_{\text{BW}}$ /pBAD-Myc-HisA	This study
<i>E. coli</i> $\Delta\text{yggS}_{\text{BW}}$ ^{-pdxH}	$\Delta\text{yggS}_{\text{BW}}$ /pBAD-AHASII	This study
<i>E. coli</i> $\Delta\text{yggS}_{\text{BW}}$ ^{-pCA24N}	$\Delta\text{yggS}_{\text{BW}}$ /pBAD-PdxH	This study
<i>E. coli</i> $\Delta\text{yggS}_{\text{BW}}$ ^{-thrC}	$\Delta\text{yggS}_{\text{BW}}$ /pCA24N	This study
<i>E. coli</i> $\Delta\text{metA}_{\text{BW}}$ ^{-pu0}	$\Delta\text{yggS}_{\text{BW}}$ /pCA24N-thrC	This study
<i>E. coli</i> $\Delta\text{metA} \Delta\text{yggS}_{\text{BW}}$	$\Delta\text{metA}_{\text{BW}}$ /pU0	This study
<i>E. coli</i> $\Delta\text{thrB}_{\text{BW}}$ ^{-pu0}	$\Delta\text{metA} \Delta\text{yggS}_{\text{BW}}$ /pU0	This study
<i>E. coli</i> $\Delta\text{thrB} \Delta\text{yggS}_{\text{BW}}$ ^{-pu0}	$\Delta\text{thrB}_{\text{BW}}$ /pU0	This study
<i>E. coli</i> $\Delta\text{thrC}_{\text{BW}}$ ^{-pu0}	$\Delta\text{thrB} \Delta\text{yggS}_{\text{BW}}$ /pU0	This study
<i>E. coli</i> $\Delta\text{thrC} \Delta\text{yggS}_{\text{BW}}$ ^{-pu0}	$\Delta\text{thrC}_{\text{BW}}$ /pU0	This study
<i>E. coli</i> $\Delta\text{thrB}_{\text{BW}}$ ^{-GFP+}	$\Delta\text{thrC} \Delta\text{yggS}_{\text{BW}}$ /pU0	This study
<i>E. coli</i> $\Delta\text{thrB} \Delta\text{yggS}_{\text{BW}}$ ^{-GFP+}	$\Delta\text{thrB}_{\text{BW}}$ /pBAD-GFP	This study
<i>E. coli</i> $\Delta\text{thrC}_{\text{BW}}$ ^{-GFP}	$\Delta\text{thrB} \Delta\text{yggS}_{\text{BW}}$ /pBAD-GFP	This study
<i>E. coli</i> $\Delta\text{thrC} \Delta\text{yggS}_{\text{BW}}$ ^{-GFP}	$\Delta\text{thrC}_{\text{BW}}$ /pBAD-GFP	This study
Plasmids		
pU0	pUC19 containing partial sequence of <i>yggS</i>	1
pUS	pUC19 expressing <i>YggS</i> -His	1
pCP20	Expresses yeast Flp recombinase gene	36
pKD46	Express lambda red recombinase	31
pKD13	Template plasmid	31
pBAD-GFP	pBAD18/GFP	Laboratory collection
pBAD24	pBAD24 empty vector	Laboratory collection
pBAD24-AHASII	pBAD24 containing <i>S. enterica</i> <i>ilvGM</i> (pDM1599)	Laboratory collection
pBAD-PdxH	pBAD-Myc-HisC expressing <i>E. coli</i> PdxH	This study
pCA24N	pCA24N empty vector	ASKA clone
pCA24N-thrC	pCA24N expressing ThrC	ASKA clone

amino acid extract was mixed with the same amount of o-phthalaldehyde (OPA)-N-acetyl-L-cysteine (NAC)-borate reagent and incubated for 15 min at 4°C. After the addition of 200 μ l of distilled water and centrifugation at 20,000 $\times g$ for 15 min at 4°C, the derivatives were analyzed by high-performance chromatography (HPLC) using an Hitachi HPLC system equipped with a Chromaster 5110 pump, a model 5210 auto sampler, a model 5310 column oven (Hitachi, Tokyo, Japan), and Shimadzu fluorescent detector RF-10A_{XL} (Shimadzu Corp., Kyoto, Japan). The excitation and emission wavelengths were 350 nm and 450 nm, respectively. The column oven was maintained at 28°C, and the total flow was 0.8 ml/min. A C₁₈ column (Mightysil RP-18 GP II column [4.6 by 150 mm, 3- μ m particle size]) equipped with a guard column (Kanto Chemical, Tokyo, Japan [4.6 by 5 mm, 5 μ m]) was used for analyses. Linear gradients of buffer B (10 mM Na₂HPO₄ [pH 6.5], 60% methanol) and buffer A (10 mM Na₂HPO₄ [pH 6.5], 10% [vol/vol] methanol) were used for separation of derivatives of amino acid (0 to 20 min, 0% to 23%; 20 to 25 min, 23% to 57%; 25 to 40 min, 57% to 100%).

B₆ vitamers analysis. B₆ vitamers (PLP, PNP, PMP, PL, PN, and PM) were quantified according to previously published protocols (3, 34) with slight modifications. Briefly, B₆ vitamers were extracted from the cells with 10 volumes (vol/wt) of 0.8 M HClO₄ containing 50 μ M deoxyripyridoxine (100 μ l of the HClO₄

solution for 10 mg *E. coli* wet cells). The suspension was subjected to extensive vortex mixing, and a total of 5 volumes (vol/wt) of 0.8 M K_2CO_3 solution (50 μ l for 10 mg *E. coli* cells) was added. The mixture was centrifuged, and the resultant supernatant (25 μ l) was used for the HPLC analysis employing fluorescence detection. The excitation and emission wavelengths were 328 nm and 393 nm, respectively. The B₆ vitamers were separated by the use of an octadecylsilyl (ODS) column (Cosmosil AR-II; Nacalai Tesque) (250 by 4.6 mm, 5- μ m particle size) using a gradient program. Mobile phase A (33 mM phosphoric acid and 8 mM 1-octanesulfonic acid, adjusted to pH 2.2 with KOH) and mobile phase B (80% acetonitrile [vol/vol]) were used for separation. The total flow rate was 0.8 ml/min. The gradient program (linear gradient) used was as follows: 0% mobile phase B to 1% B for 5 min, 1% B to 19% B for 5 min, 19% B to 28% B for 10 min, and 28% B to 63% B for 5 min. A postcolumn reagent (1 g/liter sodium bisulfite–1.0 M potassium phosphate buffer [adjusted to pH 7.5 with KOH], 0.3 ml/min) was used to enhance PLP fluorescence.

ThrC assay. Cell-free extracts were prepared using mid-log-phase cells grown in the M9 medium at 30°C. The cells (50 mg) were resuspended in 10 volumes of a disruption buffer (500 μ l) containing 100 mM potassium phosphate, 0.5 mM dithiothreitol, and 20% glycerol (pH 7.5). The cell suspension was sonicated and centrifuged (20,000 $\times$ g, 20 min, 4°C), and the resultant clear lysate was dialyzed. The protein concentration in the sample was determined by the procedure of Bradford with a Bio-Rad protein assay kit (Bio-Rad) using bovine serum albumin as the standard. ThrC activity was measured as previously described (35). Briefly, the cell extract was added to a solution containing 50 mM HEPES-NaOH (pH 8.0), 100 mM $(NH_4)_2SO_4$, 5 μ M PLP, and 1 mM P-Hse in a total volume of 100 μ l. After 30 min of incubation at 37°C, the reaction was terminated by the addition of malachite green reagent solution and the inorganic phosphate liberated from P-Hse was quantified.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.00430-19>.

SUPPLEMENTAL FILE 1, PDF file, 4.6 MB.

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T.I. designed the research, performed the experiments, and wrote the manuscript. K.Y., R.H., and A.Y. performed the experiments. D.M.D. analyzed data and wrote the manuscript, and H.H. and T.Y. analyzed the data. We declare that we have no conflict of interest.

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