Identification of YbhA as the pyridoxal 5′-phosphate (PLP) phosphatase in *Escherichia coli*: Importance of PLP homeostasis on the bacterial growth

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The gene *ybhA* of *Escherichia coli* encodes a phosphatase that has an *in vitro* specificity to dephosphorylate pyridoxal 5′-phosphate (PLP or vitamin B_{6}), a co-factor for aminotransferases and other enzymes. In this study, we found that excess pyridoxal (PL) in a minimal medium resulted in excess PLP *in vivo* and growth inhibition, which was alleviated by YbhA overproduction. Conversely, the YbhA overproduction resulted in PLP shortage *in vivo* and the correlated reduction in growth rate, which was significantly negated by PL in the medium. In addition, the overproduction of a PL kinase, PdxK or PdxY, was inhibitory to cell growth only in the absence of the functional *ybhA* gene, and the growth defects were alleviated by casamino acids in the medium, which suggested that both the shortage of, and excess, PLP resulted in the disturbance of amino acid metabolism and cell growth, as revealed by a metabolome analysis.

Key Words: amino acid metabolism; *Escherichia coli*; pyridoxal 5′-phosphatase homeostasis; pyridoxal 5′-phosphate phosphatase

Abbreviations: CAA, casamino acids; HPLC, high performance liquid chromatography; IPTG, isopropyl-β-D-thiogalactopyranoside; MOPS, potassium morpholinopropane sulfonate; OD, optical density; PL, pyridoxal; PLP, pyridoxal 5′-phosphate; PM, pyridoxamine; PMP, pyridoxamine 5′-phosphate; PN, pyridoxine; PNP, pyridoxine 5′-phosphate

Introduction

Pyridoxal 5′-phosphate (PLP or vitamin B_{6}) is an essential cofactor for many enzymes, such as aminotransferases, in living cells, and it is physiologically indispensable for amino acid metabolism. Presently, six PLP-related compounds, pyridoxal (PL), pyridoxine (PN), pyridoxamine (PM), pyridoxine 5′-phosphate (PNP), pyridoxamine 5′-phosphate (PMP) and PLP, are collectively referred to as vitamin B_{6} vitamers, which can be mutually interconverted *in vivo* (Fig. 1). However, only PLP is active as the enzymatic cofactor. While animal cells are unable to synthesize any PLP-related compound, bacteria, plants and fungi have *de novo* deoxyxylulose 5-phosphate (DXP)-dependent and DXP-independent PLP biosynthesis pathways (Fitzpatrick et al., 2007). In *Escherichia coli*, only the DXP-dependent pathway exists, in which seven enzymes compose the PLP biosynthesis pathway from DXP and erythrose 4-phosphate (Fitzpatrick et al., 2007). In addition, *E. coli* has two salvage pathways that obtain PLP from PL, PN or PM. One consists of two PL kinases, encoded by PdxK and PdxY, to synthesize PLP from PL, and another consists of PdxK and a PLP oxidase, PdxH, which synthesizes PLP from PN or PM (Mizote and Nakayama, 1989; Roseann and Walter, 1970; Yang et al.,...
The was inhibited by PLP in that forms ribulose 5-phosphate from 6-phosphogluconate PLP have been limited. It was previously shown that the toxic effects and the underlying mechanism by excess tigated (Gospe, 2009; John, 1995). However, studies on the $\cdot$ pyridoxine 5 $\cdot$ pyridoxamine, PN; pyridoxine, PMP; pyridoxamine 5 1995). Abbreviations: G3P; glycerol 3-phosphate, PL; pyridoxal, PM; pyridoxine 5-phosphate. PLP was the lowest among the examined substrates. How- ever, we were not determined whether YbhA dephosphorylates PLP and contributes to the PLP homeostasis. Here, we studied the physiological role of the ybhA gene, and we present evidence that this gene is involved in PLP homeostasis as the PLP phosphatase, and that not only a shortage of, but also an excess of, PLP is deleterious for amino acid metabolism and cell growth.

**Materials and Methods**

**Bacterial strains and plasmids.** *E. coli* BW25113 (W3110 lacI^q^ rrnBT14 ΔlacZWJ16 hsdR514 ΔaraBADΔE73 ΔrhaBADΔE74), and its derivatives, JW7049 (Δybha::Km^q^), was obtained from the *E. coli* Stock Center (National Bio-Resource Center, Mishima, Japan) (Baba et al., 2006). The Km^q^ cassette in JW7049 was removed using pCP20 to obtain RS0749 (ybha null mutant) (Cherepanov and Wackernagel, 1995; Dansker and Wanner, 2000). The ybhA^q^ allele was transferred from JW5896 to RS0749 by P1 transduction by selecting the neighboring ybhD::Km^q^ allele to make RS5896 (ybha^q^, Δybhc::Km^q^). Plasmids pCA24NΔgfp-empty, pCA24NΔgfp-ybha, pCA24NΔgfp-pdxK and pCA24NΔgfp-pdxY were obtained from the ASKA library (Kitagawa et al., 2005).

**Medium and growth conditions.** Cells were grown in MOPS minimal medium (Neidhart et al., 1974) supplemented with glucose (0.2%) at 30°C under constant shaking at 150 rpm in a test tube. Pre-cultures, 3 mL of MOPS medium with glucose (0.2%) was used, and 1/500 volume of an overnight culture (24 h) was transferred into the fresh medium for the experiments. Depending on the experiment, 0.2% casamino acids, 0 µg mL^-1^ kanamycin, or 20 µg mL^-1^ chloramphenicol, was added to the medium. Cell growth was monitored by measuring the optical density at 600 nm (OD$_{600}$) every 2 h using an OD-Monitor C & T instrument (TAITEC, Saitama, Japan). For the plating assay, MOPS minimal plates containing 0.2% glucose and 500 µM IPTG were used, and growth was observed after incubating for 24 h at 30°C.

**Quantification of PLP and metabolome analysis.** BW25113 strain was inoculated in 100 mL of MOPS minimal medium containing 0.2% glucose until the log phase (OD$_{600}$ of 0.5). To analyze the effects of PL addition, 2 mM PL was added 1 h before sampling. Cells were collected on a membrane filter by vacuum filtration. Cells on the membrane filter were quickly washed with MilliQ water and then immersed in 2 mL of methanol and briefly sonicated. A 1.6 mL portion of the cell suspension was mixed with an equal volume of chloroform and 640 µL of MilliQ water. After vortexing and centrifugation, the aqueous layer was recovered from macromolecules, and used for the following analyses. For HPLC analysis for the quantification of PLP, the aqueous layer was used for derivatization using a commercial fluorescence detection kit (Immundiagnostik, Bensheim, Germany). PLP was detected using fluorescence (excitation 320 nm, emission 415 nm). The separation was performed with the Nexera X2 HPLC system (Shimadzu, Kyoto, Japan) equipped with a prontoSIL EuroBOND C-18 column (125 x 0.4 mm, 0.5 µm particle size; BISCHOFF CHROMATOGRAPHY,

**Fig. 1. De novo synthesis and salvage pathway for PLP in *E. coli.* The de novo PLP synthesis pathway contains seven enzymes from erythrose 4-phosphate (E4P) and 1-deoxy-d-xylulose-5-phosphate (DXP). The salvage pathway for PLP is established by pdxK, pdxY, and pdxH catalyzing the conversion of PL/PN/PM into PLP (Fitzpatrick et al., 2007). The white arrow indicates the dephosphorylation of PLP into PL, which is proposed in this study. PdxH and PdxK are subjected to feedback inhibition by PLP (Ghatge et al., 2012; Zhao and Winkler, 2007). The white arrow indicates the dephosphorylation of PLP into PL, which is proposed in this study. PdxH and PdxK are subjected to feedback inhibition by PLP (Ghatge et al., 2012; Zhao and Winkler, 1995). In addition, the PLP phosphatase that counteracts the PL kinase may be a potential mechanism to prevent overproduction. The feedback mechanism includes PLP-dependent inhibition of PdxK (PL/PN/PM kinase) which is involved in the salvage pathway (Fig. 1) (Ghatge et al., 2012; Roseann and Walter, 1970; Zhao and Winkler, 1995). In addition, the PLP phosphatase that counteracts the PL kinase may be a potential mechanism in homeostasis. Consistently, a PLP-specific phosphatase is involved in the PLP homeostasis in mammalian cells (Fonda, 1992; Gao and Fonda, 1994), and a potential PLP phosphatase activity occurs in the cytoplasm of *E. coli* (Yang et al., 1998).

The YbhA protein in *E. coli* has a phosphatase activity for PLP, erythrose-4-phosphate, fructose-1,6-bisphosphate, flavin mononucleotide, thiamine-pyrophosphate, glucose-6-phosphate and ribose-5-phosphate in vitro (Kuznetsova et al., 2006), in which the enzyme Michaelis constant for PLP was the lowest among the examined substrates. However, it was not determined whether YbhA dephosphorylates PLP and contributes to the PLP homeostasis. Here, we studied the physiological role of the ybhA gene, and we present evidence that this gene is involved in PLP homeostasis as the PLP phosphatase, and that not only a shortage of, but also an excess of, PLP is deleterious for amino acid metabolism and cell growth.
Leonberg, Germany). The standard for PLP was purchased from Sigma-Aldrich. For metabolome analysis, the aqueous layer was recovered and filtrated using Ultrafree-MC ultrafilter devices for metabolome analysis. After the drying of the filtrate, the residue was dissolved in 25 μL of MilliQ water and subjected to a capillary electrophoresis time-of-flight MS (CE-TOF-MS) analysis. A metabolome analysis was conducted as described by Saito et al. (2009).

**Results**

**Excess PLP caused inhibition of the growth initiation in E. coli**

Adding PLP into the medium would have been convenient for estimating the effects of excess PLP on *E. coli* growth; however, it was previously shown that PLP was not imported into the *E. coli* cells, while PLP-related compounds (PL, PM or PN) were imported (Dempsey and Pachler, 1966). Thus, the *E. coli* wild type strain BW25113 was cultivated for 24 h in MOPS minimal medium, and the growth was monitored after a 500-fold dilution in the same medium containing 2 mM of a PLP-related compound (PL, PM or PN). As the result, a growth inhibition was observed only in the presence of PL (Fig. 2(A)). PL is converted into PLP in a one-step reaction by either PdxK, which is a kinase that commonly phosphorylates PL, PM and PN, or PdxY, which is a kinase that is specific for PL (Fig. 1). However, the conversion of PM or PN to PLP requires two steps: the sequential phosphorylation and oxidation by PdxK and PdxH (PNP/PMP oxidase). The feedback regulation of PdxK and PdxH by PLP occurs and may explain why the addition of PM or PN has no effect (Ghatge et al., 2012; Zhao and Winkler, 1995). Therefore, in this study, we used PL as a substrate to induce an excess PLP condition. In fact, the intracellular PLP accumu-

![Fig. 2.](image-url) **Supplementation of PL in the culture medium results in the inhibition of growth initiation and the accumulation of PLP in vivo.**

The MOPS minimal medium with 0.2% glucose was used as the standard medium in all panels. (A) Growth curve of *E. coli* wild type strain (BW25113) in the presence of 2 mM each of PL (squares), PM (diamonds) and PN (triangles), respectively, or without any supplementation, as the control (circles). The optical density (OD_{600}) was measured every 2 h and plotted. (B) Chromatogram of HPLC analysis in *E. coli* wild type strain supplemented with (black) or without (gray) 2 mM PL. (C) Quantification of PLP from HPLC analysis. Error bars represent the standard error. The asterisk represents a statistically significant change compared with the supplementation with, or without, 2 mM PL calculated by a Student’s t test for \( P < 0.01 \). (D) Growth curve of *E. coli* wild type strain (BW25113). Result from additions of 0 (circles), 1 (triangles), 1.5 (diamonds), 2 (squares) or 2.5 (asterisks) mM of PL are indicated. The optical density (OD_{600}) was measured every 2 h and plotted. The results are the average data of three independent experiments.

![Fig. 3.](image-url) **YbhA could act as a PLP phosphatase in vivo.**

The MOPS minimal medium with 0.2% glucose was used. The optical density (OD_{600}) was measured every 2 h and plotted. Growth curves of the wild type strain transformed with pCA24N_gfp-empty (circles), pCA24N_gfp-ybhA (squares), pCA24N_gfp-cof (diamonds) and pCA24N_gfp-yigL (triangles). 2 mM PL was supplemented into each culture with, or without, 500 μM IPTG (closed or open symbols, respectively). These results are the average data of three independent experiments.
Role of *E. coli* YbhA in PLP homeostasis

The MOPS minimal medium with 0.2% glucose was used as the standard medium in all of the panels. The optical density (OD_{600}) was measured every 2 h and plotted in (A) and (C). (A) Growth curves of the wild type strain transformed with pCA24N_{Δgfp-empty} (open circles or triangles) and pCA24N_{Δgfp-ybhA} (open squares, closed symbols and asterisks). IPTG was supplemented at 0 (open circles and squares), 75 (asterisks), 125 (closed diamonds) or 500 (open triangles and closed squares) μM. (B) Intracellular concentration of PLP against growth rate of pCA24N_{Δgfp-ybhA}. IPTG was supplemented at 0 (open square), 75 (asterisk) or 500 μM (squares). The numbers (1–4) indicate the sampling points shown in (A). Error bars represent standard error resulted from three independent experiments. The absence of an error bar indicates that the standard error was negligible. (C) Growth curves of the wild type strain transformed with pCA24N_{Δgfp-ybhA} with the addition of 500 μM IPTG and 0 (closed squares), 0.1 (closed circles), 1 (closed triangles) or 2.5 (closed diamonds) mM of PL. No supplementation is indicated with open squares. These results are the average data of three independent experiments.

Overexpression of YbhA results in the reduction of the growth rate and the shortage of PLP in vivo.

The appearance of visible growth was delayed in a PL concentration-dependent manner, while the doubling time after the onset of the visible growth was not affected by the PL concentration (Fig. 2(D)). Thus, the excess PLP accumulation appeared to cause inhibition of the growth initiation.

Identification of YbhA as the pyridoxal 5′-phosphate phosphatase in vivo

If the inhibition of growth initiation observed under excess PL conditions actually resulted from the increased PLP in the cell, the overexpression of phosphatases specific for PLP should alleviate the inhibition. To examine this possibility, three phosphatases, Cof, YbhA and YigL, that dephosphorylate PLP *in vitro* (Kuznetsova et al., 2006), were overproduced in BW25113 using ASKA library clones under the control of P_{}^{P_{T5-lac}} promoter (Kitagawa et al., 2005), and the effects on the PL-induced inhibition of growth initiation were investigated. Only YbhA overproduction alleviated the inhibitory effects caused by excess PL (Fig. 3). It was confirmed that YbhA, Cof and YigL proteins were expressed at the similar level by immunoblot analysis using his-tag specific antibody (Fig. S1). Thus, these results indicated that only YbhA could function as a PLP phosphatase *in vivo*.

Shortage of PLP caused reduction of the growth rate in *E. coli*

Giving that YbhA is a PLP phosphatase, an excess *ybhA* induction would cause PLP shortage *in vivo* and result in the growth inhibition. To examine this, a YbhA expression strain was cultivated in MOPS medium containing various concentrations of IPTG (0–500 μM), and the cell growth was monitored. The growth rate of the YbhA expression strain was reduced in an IPTG concentration-dependent manner (Fig. 4(A)). To further understand the correlation between the PLP concentration and the growth rate, we then measured the intracellular PLP concentration. As the result, approximately 400 pmol/mg of PLP was found from cells growing without IPTG as well as from cells after visible growth in the presence of 500 μM of IPTG (Figs. 4(A) and (B)). In contrast, significant reduction of PLP concentration was observed from the growth-inhibited cells with 500 μM of IPTG. A slightly higher PLP concentration was found in the cells supplemented with 75 μM IPTG than in the growth-inhibited cells supplemented with 500 μM IPTG. These results indicated that the shortage of PLP resulted in a reduction of the growth rate.

Next, the YbhA expression strain was cultured in a MOPS medium containing 500 μM of IPTG supplemented with various concentrations of PL (0–2.5 mM), and the cell growth was monitored. The inhibitory effect on cell growth by the induction of YbhA was conversely alleviated by the PL addition to the medium in a PL concentration-dependent manner (Fig. 4(C)). Thus, it was indicated that the PL addition to the medium caused excess PLP *in vivo*, and, conversely, the overproduction of *ybhA* caused a PLP shortage, and both the excess and shortage of PLP *in vivo* had inhibitory effects mainly on the cell growth initiation and the growth rate, respectively.

Fig. 4. Overexpression of YbhA results in the reduction of the growth rate and the shortage of PLP *in vivo*.

The MOPS minimal medium with 0.2% glucose was used as the standard medium in all of the panels. The optical density (OD_{600}) was measured every 2 h and plotted in (A) and (C). (A) Growth curves of the wild type strain transformed with pCA24N_{Δgfp-empty} (open circles or triangles) and pCA24N_{Δgfp-ybhA} (open squares, closed symbols and asterisks). IPTG was supplemented at 0 (open circles and squares), 75 (asterisks), 125 (closed diamonds) or 500 (open triangles and closed squares) μM. (B) Intracellular concentration of PLP against growth rate of pCA24N_{Δgfp-ybhA}. IPTG was supplemented at 0 (open square), 75 (asterisk) or 500 μM (squares). The numbers (1–4) indicate the sampling points shown in (A). Error bars represent standard error resulted from three independent experiments. The absence of an error bar indicates that the standard error was negligible. (C) Growth curves of the wild type strain transformed with pCA24N_{Δgfp-ybhA} with the addition of 500 μM IPTG and 0 (closed squares), 0.1 (closed circles), 1 (closed triangles) or 2.5 (closed diamonds) mM of PL. No supplementation is indicated with open squares. These results are the average data of three independent experiments.
The ybhA mutant was sensitive to the over-expression of pyridoxal kinase

To estimate the physiological role of the ybhA gene, we first compared the growth of the wild type and the ybhA mutant strain on a MOPS minimal medium plate containing increasing concentrations of PL (0–2.5 mM); however, no difference between the two strains was observed (data not shown). Subsequently, expression vectors of pyridoxal kinase (pCA24N Δgfp-pdxK and -pdxY from the ASKA library) were examined to induce PLP accumulation in vivo. These plasmids were used to transform BW25113 and JW0749 (Δybhd::KmR), and the transformants were streaked on MOPS minimal medium plates containing 500 μM of IPTG and incubated for 24 h. For BW25113 derived strains, the expression of PdxK or PdxY had no effect on the growth (Fig. 5(A)). However, the over-expression of PdxK or PdxY in JW0749 resulted in significant growth defects, while PdxK was more inhibitory than PdxY (Fig. 5(B)). Introduction of the ybhA+ allele into RS0749 (Δybhd::KmR) by P1 transduction resulted in the disappearance of the IPTG-sensitive phenotype (Fig. 5(C)). These results again supported that ybhA encodes a PLP phosphatase.

Supplementation of the medium with casamino acids cancelled the growth inhibition caused by both the excess and shortage of PLP

To understand the underlying mechanism of the observed growth inhibition by an excess or shortage of PLP, we performed a growth assay with supplemental casamino acids, because PLP is an essential cofactor for enzymes involved in amino acid metabolism. First, to examine the effects of the addition of casamino acids on growth inhibition by excess PL, the wild type strain was cultivated for 24 h in a MOPS minimal medium and in a 1/500 dilution of the same medium containing 500 μM of IPTG and 0.2% of casamino acids. The over-expression of PdxK or PdxY is indicated as PdxKωex or PdxYωex, respectively. These results are representative data of three independent experiments.
Role of *E. coli* YbhA in PLP homeostasis

PLP accumulation alters amino acid profiles

To ensure the metabolic effects of PLP accumulation *in vivo*, we performed a metabolome analysis of cells incubated with or without PL. The wild type strain was inoculated in duplicates in the MOPS minimal medium until the log phase (OD$_{600}$ of 0.4). To observe the effects of the addition of PL, 2 mM of PL was added to one portion and the cultivation continued for another hour. Cells were collected, intracellular metabolites were measured and the results were compared between samples with or without PL. Alteration of intracellular concentrations of amino acids were observed, as arginine, asparagine, and tyrosine were reduced and proline, histidine and glutamate were increased (Fig. 7). Thus, excess PLP may affect amino acid metabolism, resulting in amino acid imbalance and the inhibition of growth initiation.

**Discussion**

PLP is an essential cofactor for a large number of enzymes involved in amino acid and other metabolisms, and, in this line, the pathological aspects of PLP shortage on mammalian cells have been well documented (Ghatge et al., 2016; Gospe, 2009). In this study, we consistently observed the inhibitory effect of PLP shortage as revealed by the slow growth phenotypes (Fig. 4). Because these effects were largely negated by the addition of casamino acids in the medium, the effects likely resulted from an insufficient supply of available amino acid pools.

Other than the effect of PLP shortage, we found that an excess of PLP also has a negative effect on bacterial growth. Inhibitory effects of excess PLP on enzymatic reactions were reported in previous biochemical studies (Ohsawa and Gualerzi, 1981; Vermeersch et al., 2004), and the authors speculated on the involvement of non-specific damaging of cellular molecules by the reactive aldehyde group of PLP. In this study, we observed that excess PLP specifically resulted in the inhibition of growth initiation (Fig. 2). These inhibitions were negated by the addition of casamino acids as is the case of PLP shortage (Figs. 5 and 6), and metabolome analysis indicated that the excess PLP resulted in the imbalance of intracellular amino acid pools (Fig. 7). Thus, the inhibition of some enzyme(s) for amino acid metabolism and the resultant decreased levels of some particular amino acids appear to be the reason for the growth defect because of the restoration by casamino acids addition. Meanwhile, it should be noted that the initiation of cell growth appeared to have been most significantly affected, which is different from the case of PLP shortage. This could be explained if arginine, asparagine and/or tyrosine, which showed a reduced concentration (Fig. 7), play a specific role in the growth initiation, while further analysis is required to examine this hypothesis. Importantly, this is the first report which describes the inhibitory effect of PLP excess and proposes an underlying mechanism in living cells.
To maintain the PLP homeostasis, the final enzymes involved in PLP biosynthesis, PdxK and PdxH, are directly inhibited by PLP in *E. coli* (Ghatge et al., 2012; Zhao and Winkler, 1995). In addition, PLP phosphatase, which dephosphorylates PLP and decreases the PLP concentration, has been considered to be another mechanism that maintains PLP homeostasis. Here, we have shown that YbhA could alleviate the inhibitory effect of excess PLP in vivo (Fig. 3), and, also, we have shown that overproduction of YbhA caused a shortage of PLP in vivo and a reduction of growth rate (Figs. 4(A) and (B)). Thus, we propose that YbhA contributes to the PLP homeostasis as a PLP phosphatase. In addition to PLP, YbhA dephosphorylates fructose 1,6-bisphosphate in vitro, and, if this works in vivo, it may function in gluconeogenesis (Kuznetsova et al., 2006). To examine this, we estimated the fructose 1,6-bisphosphatase activity of YbhA using a growth assay in the *fbp* mutant JW4191 (*fbp*: Km§) that cannot grow on acetate as the sole carbon source (Donahue et al., 2000; Fraenkel et al., 1965). As the result, JW4191 harboring the YbhA-overproducing plasmid could not grow in MOPS minimal medium containing acetate as the sole carbon source (Fig. S2). Thus, we propose that YbhA does not function physiologically as fructose 1,6-bisphosphatase and that the primary role of YbhA is as a PLP phosphatase for the PLP homeostasis. This is the first report of the identification of a PLP-specific phosphatase in *E. coli*.

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Supplementary Materials

Supplementary figures are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

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