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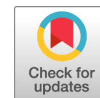
RESEARCH ARTICLE



Pyridoxal Reductase, PdxI, Is Critical for Salvage of Pyridoxal in *Escherichia coli*

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ABSTRACT Pyridoxal 5′-phosphate (PLP) is the biologically active form of vitamin B_6 and an essential cofactor in all organisms. In *Escherichia coli*, PLP is synthesized via the deoxyxylulose 5-phosphate (DXP)-dependent pathway that includes seven enzymatic steps and generates pyridoxine 5′-phosphate as an intermediate. Additionally, *E. coli* is able to salvage pyridoxal, pyridoxine, and pyridoxamine B_6 vitamers to produce PLP using kinases PdxK/PdxY and pyridox(am)ine phosphate oxidase (PdxH). We found that *E. coli* strains blocked in PLP synthesis prior to the formation of pyridoxine 5′-phosphate (PNP) required significantly less exogenous pyridoxal (PL) than strains lacking *pdxH* and identified the conversion of PL to pyridoxine (PN) during cultivation to be the cause. Our data showed that PdxI, shown to have PL reductase activity *in vitro*, was required for the efficient salvage of PL in *E. coli*. The *pdxI*$^+$ *E. coli* strains converted exogenous PL to PN during growth, while *pdxI* mutants did not. In total, the data herein demonstrated that PdxI is a critical enzyme in the salvage of PL by *E. coli*.

IMPORTANCE The biosynthetic pathway of pyridoxal 5′-phosphate (PLP) has extensively been studied in *Escherichia coli*, yet limited information is available about the vitamin B_6 salvage pathway. We show that the protein PdxI (YdbC) is the primary pyridoxal (PL) reductase in *E. coli* and is involved in the salvage of PL. The orthologs of PdxI occur in a wide range of bacteria and plants, suggesting that PL reductase in the B_6 salvage pathway is more widely distributed than previously expected.

KEYWORDS *Escherichia coli*, pyridoxal phosphate, pyridoxal reductase, pyridoxal salvage, pyridoxine, vitamin B_6

Pyridoxal 5′-phosphate (PLP) is the biologically active form of vitamin B_6 that is used as a cofactor in various enzymes. PLP-dependent enzymes catalyze diverse biochemical reactions, including transamination, racemization, decarboxylation, and α,β-elimination or replacement of chemical groups at C_β or C_γ involving amino acid, sugar, and lipid metabolisms (1, 2). Most bacteria harbor several MocR/GabR-type PLP-binding transcriptional regulators to modulate amino acid and/or vitamin B_6 metabolisms (3–6).

Two distinct pathways for PLP biosynthesis, deoxyxylulose 5-phosphate (DXP)-dependent and DXP-independent pathways, have been reported. In *Escherichia coli* and a few members of the proteobacteria, PLP is synthesized via the DXP-dependent pathway that includes seven reaction steps catalyzed by seven enzymes (GapB, PdxB, SerC, PdxA, DXS, PdxJ, and PdxH) from erythrose 4-phosphate, glyceraldehyde 3-phosphate, and pyruvate (7, 8). In this pathway, the pyridine ring is formed by the action of the pyridoxine 5′-phosphate synthase (PdxJ), which catalyzes the condensation of DXP with 3-hydroxy-1-aminoacetone phosphate to form pyridoxine 5′-phosphate (PNP) (9) (Fig. 1). PNP is oxidized by a flavin mononucleotide (FMN)-dependent enzyme pyridoxine/pyridoxamine 5′-phosphate oxidase (PdxH) to form PLP (10). PdxH can also oxidize pyridoxamine 5′-phosphate (PMP) to produce PLP.

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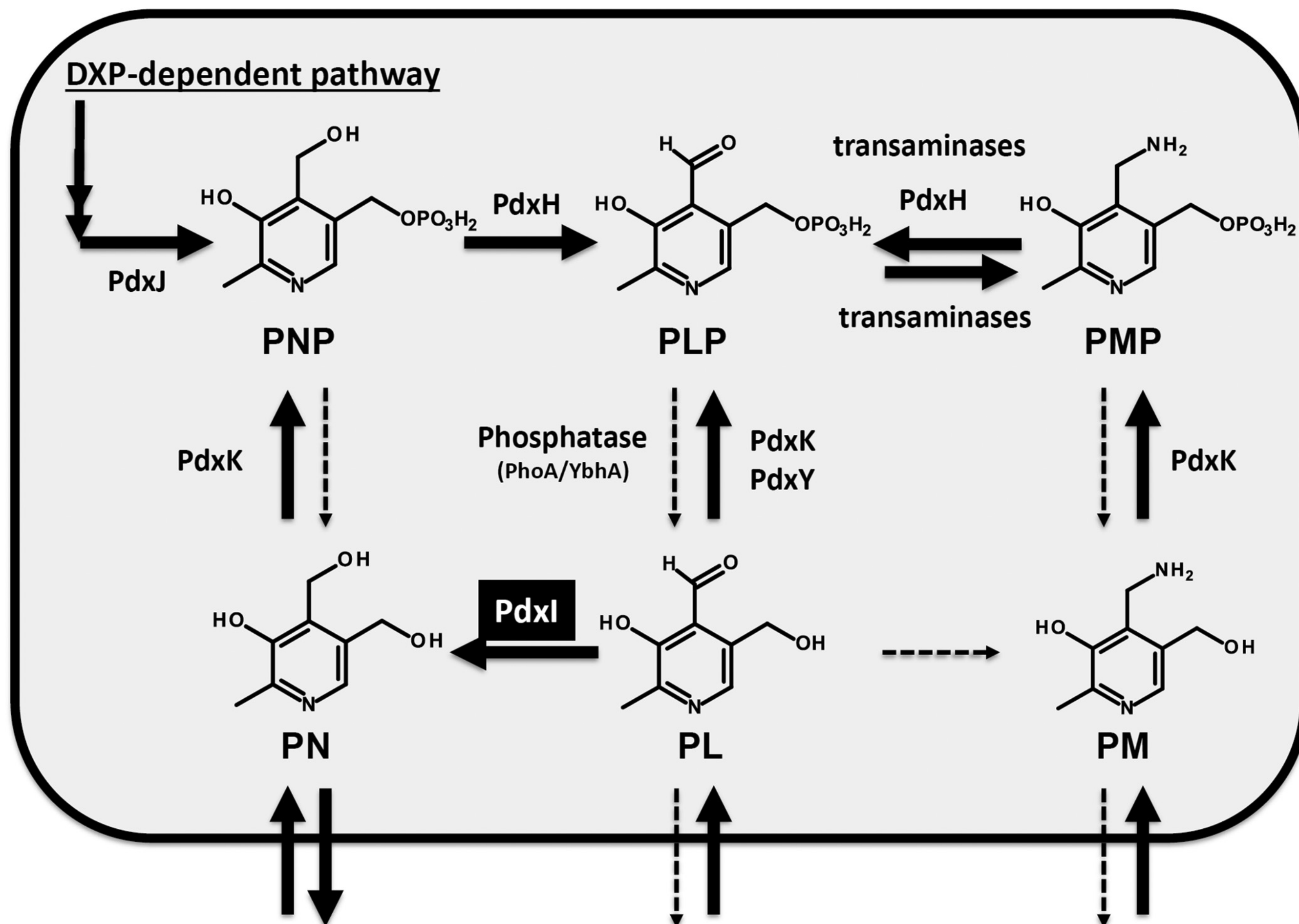

FIG 1 Vitamin B_6 salvage pathway in *E. coli*. Biosynthesis of PLP via the DXP-dependent pathway and salvage of B_6 vitamers are schematically shown. Pyridoxine 5′-phosphate synthase (PdxJ) catalyzes the condensation of DXP with 3-hydroxy-1-aminoacetone phosphate to form pyridoxine 5′-phosphate (PNP) in *de novo* synthesis. PLP can be formed from PNP or PMP by pyridoxine/pyridoxamine 5′-phosphate oxidase (PdxH). PdxK can phosphorylate the three B_6 vitamers, pyridoxine (PN), pyridoxamine (PM), and pyridoxal (PL), allowing PLP to be salvaged from the environment by the routes depicted. PdxY contributes to the phosphorylation of PL only. In addition, amino acid transaminases convert PMP to PLP and vice versa during their catalytic cycle. Some phosphatases, including YbhA and PhoA, can dephosphorylate PLP. There is currently little information about the bacterial vitamin B_6 transporter(s). This study demonstrated that PdxI contributes to PL salvage and catalyzes the irreversible conversion of PL to PN under physiological conditions. Known or identified pathways are shown in solid arrows. Broken arrows indicate unidentified/missing pathways.

E. coli can convert various forms of B_6 vitamers to PLP using the salvage pathway (11). Our current understanding of the salvage pathway in *E. coli* is summarized in Fig. 1. Unlike the biosynthetic pathway, details of the salvage pathway have not been fully elucidated. Two kinases, PdxK and PdxY, can phosphorylate the 5′ position of the B_6 vitamers (12, 13). PdxK is capable of phosphorylating all three B_6 vitamers, pyridoxine (PN), pyridoxamine (PM), and pyridoxal (PL), whereas PdxY phosphorylates only PL (13). Various amino acid transaminases are capable of converting PMP to PLP and vice versa via their reaction mechanism. Some phosphatases, including YbhA and PhoA, are capable of dephosphorylating PLP and/or PNP *in vivo* and *in vitro* (14, 15).

During the study of the biosynthesis, homeostasis, and metabolic network of vitamin B_6 in *Escherichia coli* and *Salmonella enterica* (16–18), we found that *E. coli* strains lacking *pdxJ* had different PL requirements than those lacking *pdxH*. This result was unexpected given that both lesions abolish PLP biosynthesis. The *pdxH* mutant required 10 times more PL than the *pdxJ* mutant to reach an optimal growth yield. This study was initiated to determine the cause of this difference. The data herein show that the protein YdbC (PdxI), reported to catalyze PL reductase activity *in vitro* (19), was primarily responsible for the differences of PL requirement observed and is a component of PL salvage in *E. coli*.

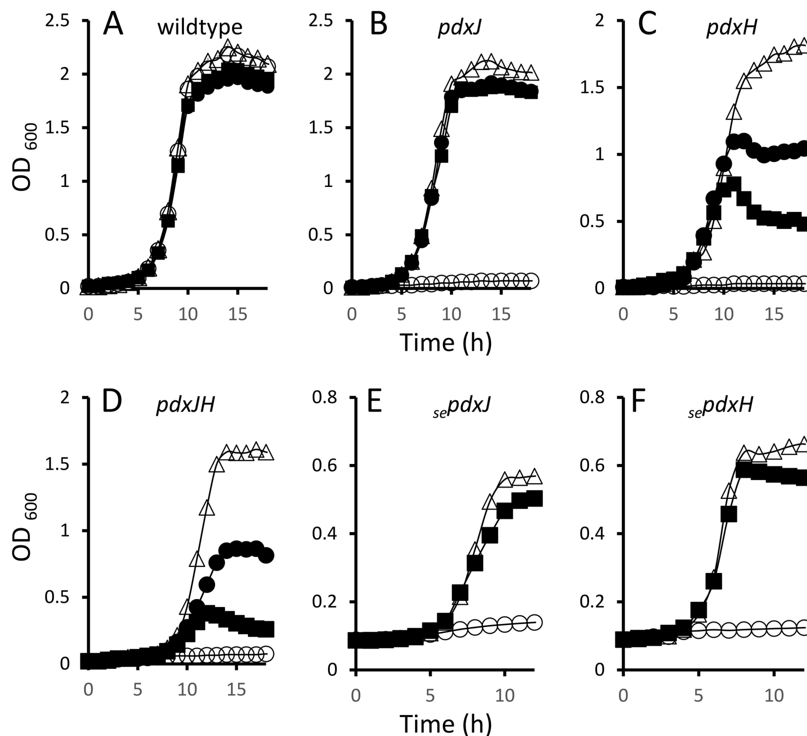

FIG 2 PL requirement of *E. coli pdxJ*, *pdxH*, or *pdxJ pdxH* mutants. (A to D) Wild-type (A) and *pdxJ* (B), *pdxH* (C), or *pdxJ pdxH* (D) mutant strains of *E. coli* were grown in M9-glucose medium in the absence (○) or presence of PL at 0.1 μM (■), 0.25 μM (●), or 1 μM (△). (E and F) *pdxJ* (E) and *pdxJ pdxH* (F) mutants of *S. enterica* were grown in NCE glucose medium in the absence (○) or presence of 0.1 μM (■) or 1 μM (△) PL. The data represent the averages from triplicate experiments. Cell growth was recorded by the OD-Monitor C&T apparatus (A to D) or the ELx808 (E and F) using a 96-well plate.

RESULTS AND DISCUSSION

Elimination of *pdxH* increases PL requirement in *E. coli*. The ability of exogenous pyridoxal (PL) to allow growth of strains of *E. coli* blocked in the synthesis of PLP has been attributed to the PL kinase activity of PdxK/PdxY (Fig. 1). In the course of other work, it was noted that *pdxJ* and *pdxH* mutants, both of which cannot synthesize PLP *de novo*, required different concentrations of PL for growth in an M9-glucose medium. The *pdxJ*-deficient strain grew with a rate and reached a final density similar to those of a wild-type strain (Fig. 2A) when 0.1 μM PL was provided (1-h doubling time) (Fig. 2B). In contrast, the *pdxH* mutant required 1 μM of PL to achieve an optimal growth yield (1-h doubling time) (Fig. 2C). The final yield, but not the growth rate, of the *pdxH* mutant was affected by the concentration of PL. This result suggested that PL was limiting and was not simply being poorly utilized. Eliminating PdxJ or PdxH blocked PLP formation, though the *pdxH* mutant accumulated PNP due to flux through the PdxJ-dependent pathway (Fig. 1). A *pdxJ pdxH* double mutant exhibited slower growth than either the *pdxJ* or *pdxH* single mutant (1.5-h doubling time) but had a growth pattern most similar to that of the *pdxH* mutant strain, requiring >10-fold more exogenous PL than the *pdxJ* mutant (Fig. 2D). These data supported the conclusion that flux through the PLP biosynthetic pathway did not contribute to the increased PL requirement of the *pdxH* mutant.

The above-described data were not anticipated based on the described salvage pathway for B_6 vitamers in *E. coli*. In contrast, *Salmonella enterica* displayed a result closer to what was expected based on the described salvage enzymes. Strains lacking *pdxJ* or *pdxH* had a growth response to 0.1 μM of PL that was more similar to the full-growth allowed by 1 μM in no-carbon essential (NCE) minimal medium (Fig. 2E and F).

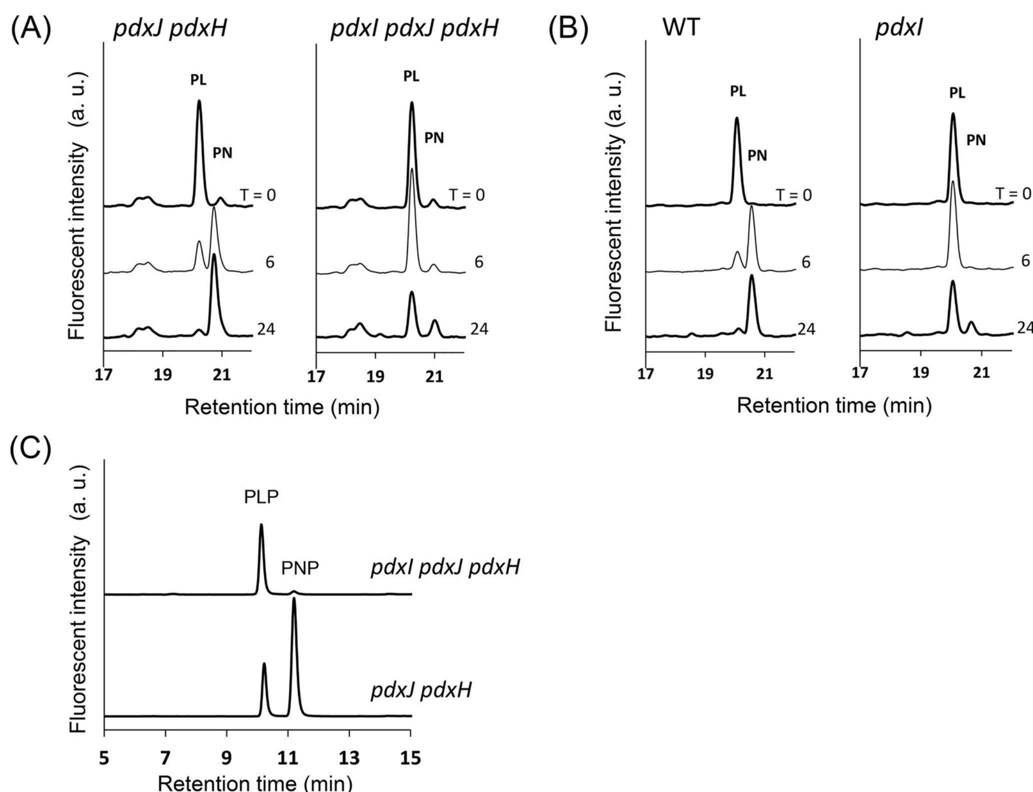

FIG 3 A *pdxI* mutation prevents conversion of extracellular PL to PN and intracellular accumulation of PNP. The *pdxJ pdxH* and *pdxI pdxJ pdxH* mutant strains of *E. coli* were grown in M9-glucose medium supplemented with 5 μM PL. The culture supernatant was collected at different time points (0, 6, or 24 h). Internal pool concentrations of B_6 vitamers were determined with cells collected at early stationary phase (OD_{600} of 0.7) as described in Materials and Methods. The *pdxJ pdxH* double mutant converted all of the extracellular PL to PN during its cultivation (A, left) and accumulated PNP in the cells (C, bottom trace). The *pdxI* mutation significantly decreased the conversion of exogenous PL to PN (A, right) and intracellular accumulation of PNP (C, top trace). A wild-type *E. coli* strain showed conversion of extracellular PL to PN during cultivation (B, left), and the *pdxI* mutation significantly decreased this conversion (B, right). Experiments were performed at least three times with similar results, and representative chromatograms are shown.

***E. coli pdxJ pdxH* mutant converts exogenous PL to PN and accumulates PNP.** Based on the kinases implicated in the salvage pathway, PdxK and PdxY, the PLP requirement of a *pdxJ* mutant should be satisfied with PL, PN, or PM (Fig. 1). This expectation was confirmed by growth analyses with 0.1 μM of each B_6 vitamer (data not shown). In contrast, a *pdxH* mutant is not expected to utilize exogenous PN or efficiently convert PM to PLP and is likely to accumulate PNP synthesized through the DXP-dependent pathway (Fig. 1). Thus, a difference in the requirement for PL in the *pdxJ* and *pdxH* mutants was not predicted *a priori*. High-performance liquid chromatography (HPLC) analyses of the growth medium showed the disappearance of PL from the medium during the growth of a *pdxJ pdxH* mutant. As shown in Fig. 3A, the M9 growth medium of the *pdxJ pdxH* mutant, which contained 5 μM PL at time zero ($T = 0$), had only PN (~4.5 μM) after 24 h of cell growth. PL was also absent in the medium after growth of the wild type (Fig. 3B) or the *pdxH* mutant strain (data not shown) and was seemingly replaced by the PN that accumulated. When cells were removed from the culture medium after 6 h of cultivation (by centrifugation and following filtration with a 0.45-μm filter membrane), no further conversion of PL to PN was observed, indicating that the conversion of PL to PN was mediated by *E. coli* cells (data not shown). The intracellular pools of B_6 vitamers were analyzed in the *pdxJ pdxH* double mutant after growth in M9-glucose medium in the presence of 5 μM PL. Despite the block in synthesis and the lack of a known pathway from PL to PNP, a significant amount of PNP accumulated in the cells (Fig. 3C, bottom trace). Consistent with the growth results described above, when an *S. enterica pdxJ pdxH* double mutant strain

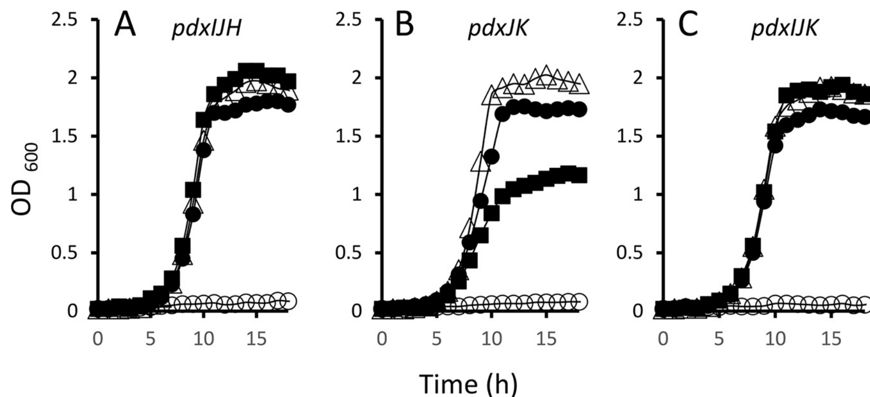

FIG 4 PL requirement of *E. coli pdxI pdxJ pdxH*, *pdxJ pdxK*, or *pdxI pdxJ pdxK* mutant. The *pdxI pdxJ pdxH* (A), *pdxJ pdxK* (B), and *pdxI pdxJ pdxK* (C) mutant strains of *E. coli* were grown in M9-glucose medium in the absence (○) or presence of 0.1 μM (■), 0.25 μM (●), or 1 μM (△) PL. The data represent the averages from biological triplicates. Cell growth was recorded by the OD-Monitor C&T apparatus.

was grown under similar conditions, PN did not appear in the medium and PNP did not accumulate in the cells (data not shown). These data suggested that *E. coli* had a mechanism(s) to convert PL→PN and/or PLP→PNP that is both internal and not present in *S. enterica*. The *pdxH* mutation abolishes the utilization of PN and PNP as a PLP source in *E. coli* (Fig. 1). If PL were partially converted to PN or PNP during growth, it could explain the increased PL requirement observed with the *pdxH* or *pdxJ pdxH* mutant of *E. coli*, and PL availability would be decreased (Fig. 1).

PdxI is required for the conversion of PL to PN in *E. coli*. The presence of a PL reductase in *E. coli* would explain these data. A recent global study using a supplemented metabolome extract and purified protein identified a protein that catalyzes the NADPH-dependent reduction of PL to PN (19). The relevant protein, YdbC, contains 286 amino acids, belongs to the aldo-keto reductase (AKR) family (20), and was renamed PdxI based on the activity. The same protein had reductase activity with 5-nitro benzisoxazole→2-cyano-4-nitrophenol with the K_m and k_{cat} values of 270 μM and 0.11 s^{-1}, respectively (21), and neither activity was attributed a physiological role.

A working model suggested that PdxI functioned as a PL reductase and converted PL to PN in the growth medium of *E. coli* strains. An insertion/deletion of *pdxI* was constructed and transduced into relevant genetic backgrounds. The requirement for B_6 vitamers was determined in the relevant strains. As expected based on the working model, the *pdxJ pdxH pdxI* triple mutant (DM16865) had a lower requirement for PL in M9-glucose medium, and 0.1 μM PL in the medium allowed full growth (Fig. 4A). The *pdxH pdxI* double mutant had a similarly low requirement for PL compared to that of the parental *pdxH* strain (data not shown). Together, these data showed that the increased requirement for PL was dependent on PdxI. Consistently, HPLC analyses of the vitamin B_6 pool showed that *pdxI* lesion in the *pdxJ pdxH* mutant background prevented the accumulation of PNP inside the cells (Fig. 3C). In strains with a lesion in *pdxI* (*pdxI pdxH* or *pdxI pdxJ pdxH* mutant), PL was not converted to PN over time in the growth medium (Fig. 3A). Finally, in an otherwise wild-type background, a *pdxI* mutation significantly decreased the accumulation of PN in medium supplemented with exogenous PL (Fig. 3B). A similar involvement of PL reductase in the accumulation of PN in the growth medium was reported in yeasts (22).

The physiological significance of the rapid PdxI-dependent conversion of PL to PN during growth and the location of this conversion remain to be elucidated. After 24 h of cultivation in M9 medium supplemented with PL, the *pdxI* mutant strain accumulated a small amount of PN in the medium (Fig. 3A and B). This result suggests there might be an additional enzyme(s) capable of reducing PL in *E. coli*. The genome of *E. coli* K-12 encodes PdxI and eight other proteins that belong to the AKR superfamily (DkgA [15%], DkgB [19%], YdjG [21%], YgdS [tas] [16%], YdhF [15%], YghZ [17%], YeaE

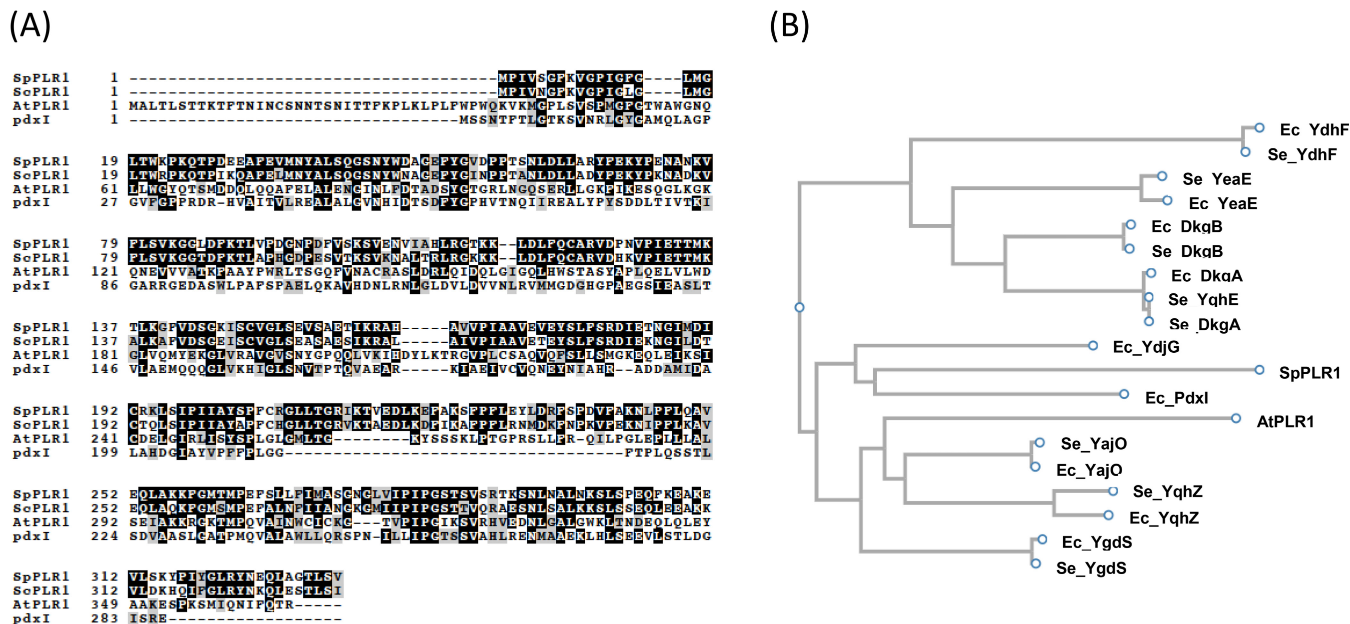

FIG 5 Sequence alignment of PL reductases. (A) Primary sequences of PL reductase of *E. coli* (PdxI), *S. pombe* (SpPLR1), *S. cerevisiae* (ScPLR1), and *A. thaliana* (AtPLR1) were aligned using the ClustalW program (https://www.genome.jp/tools-bin/clustalw). *E. coli* PdxI exhibits weak similarity to the three eukaryotic PL reductases. The PdxI is 13.6%, 15.0%, and 15.4% identical to the *S. pombe* PLR1, *S. cerevisiae* PLR1, and *A. thaliana* PLR1, respectively. (B) A phylogenetic tree of PdxI, SpPLR1, AtPLR1, and other proteins belonging to the AKR superfamily of *E. coli* and *S. enterica* based on the ClustalW algorithm is shown.

[15%], YajO [20%], and PdxI; the percent values in brackets indicate sequence identities to PdxI). In yeasts and plants, members of the AKR family have PL reductase activity (22–25). Alignment analyses showed that the *E. coli* PdxI shares weak homology with the eukaryotic PL reductases and is 13.6%, 15.0%, and 15.4% identical to the *Schizosaccharomyces pombe* PLR1, *Saccharomyces cerevisiae* PLR1, and *Arabidopsis thaliana* PLR1, respectively (Fig. 5A). The phylogenic analysis suggested that PdxI is evolutionarily related to PL reductases of yeast, although their amino acid identities are low. In contrast, PdxI is evolutionarily less related to the PL reductase from *A. thaliana* (Fig. 5B). BLAST analysis using PdxI sequence as a query sequence identified proteins exhibiting greater than 30% identities to PdxI in many bacteria and plants, including *Shigella sonnei*, *Streptomyces coelicolor*, *Deinococcus radiodurans*, *Thermotoga maritima*, *Pseudomonas aeruginosa*, *Glycine max*, and *A. thaliana*. The BLAST search also showed that most *E. coli* strains, excluding *E. coli* B strains such as BL21, have a PdxI ortholog that has >90% identity. No protein-encoding gene that exhibits >30% identity to *E. coli* PdxI was found in the genome of *S. enterica* LT2 strain and most of other *S. enterica* strains, while there were 8 members of the AKR superfamily (YdjG is missing, while YqhE is present in the *S. enterica* strain) (Fig. 5B), supporting the conclusion that PdxI is the primary enzyme that efficiently converts PL to PN in *E. coli*.

Kinetic parameters of PdxI. Our growth data suggested that in *E. coli pdxH* and *pdxJ pdxH* mutant strains, the flux to PLP from direct salvage (PL to PLP) via kinases PdxK and PdxY (12, 13) is lower than flux via a salvage pathway requiring reduction of PL by PdxI (PL→PN→PNP→PLP). PL reduction catalyzed by PdxI was kinetically characterized using the purified enzyme. PL showed Michaelis-Menten kinetics in the substrate concentration range examined (0 to 0.5 mM). The apparent K_m and k_{cat} values of the PL reductase activity in the presence of 0.3 mM NADPH were determined to be 31 ± 3 μM and 84 ± 2 s^{-1}, respectively. PdxI had no detectable reductase activity with either PLP or PM as the substrates under the assay conditions used. Pyridoxal reductases of *S. cerevisiae* and *S. pombe* catalyze the reverse reaction (conversion of PN to PL in the presence of $NADP^+$), although the efficiencies are low (22, 24). This activity was not detected for PdxI.

TABLE 1 Effect of *pdxI* deletion on intracellular B_6 pool

Genotype[a]	Amt (pmol/mg [wet wt] cells) of[b]:		
	PLP	PNP	PMP
WT	52 ± 7	ND	30 ± 4
pdxI	48 ± 2	ND	25 ± 3

[a]Wild-type (WT) and *pdxI* mutant were grown in M9-glucose medium.
[b]The intracellular B_6 pool was determined as described in Materials and Methods. The *pdxI* mutation did not affect the levels of intracellular B_6 vitamers. ND, no detectable amount was observed. The data represent the averages and standard deviations from triplicate experiments.

The K_m and k_{cat} values for the kinase activity of PL catalyzed by PdxK are reported to be 60 μM and 4 s^{-1}, respectively (26). PdxY exhibits only 1% of the kinase activity compared to that of PdxK (27, 28). The expression levels of PdxI, PdxK, and PdxY were not expected to be significantly different (29). In total, these data support the hypothesis that the conversion of PL to PN (by PdxI) is favored over the conversion of PL to PLP (by PdxK and/or PdxY) in *E. coli* cells. The efficient conversion of PL to PN would result in a rapid shortage of PL in the medium, causing the increased requirement for PL in the *pdxH* mutants, since these strains are unable to use the PN or PNP that would result from PdxI and PdxK activity.

Contribution of PdxI in the B_6 salvage pathway. In *S. pombe*, the deletion of the pyridoxal reductase gene (*plr1*) resulted in a decrease in total vitamin B_6 and PMP contents (22). The transfer DNA (T-DNA) insertion mutant in the pyridoxal reductase in *A. thaliana* exhibits significantly lower levels of total B_6 vitamer, PL, PLP, PM, and PMP (25). We investigated the influence of disruption of *pdxI* on the homeostasis of B_6 vitamers. The wild type and the *pdxI* mutant were grown in M9-glucose medium, and their intracellular B_6 pools were analyzed. Importantly, the growth of the *pdxI* mutant was not significantly different from that of the wild-type strain when they were cultivated in M9-glucose medium. PL did not accumulate in either of the strains. The result showed that lack of *pdxI* did not significantly impact the intracellular B_6 pools in *E. coli* under the condition examined (Table 1).

Conclusions. Here, we report that *E. coli* possesses an efficient pathway to convert PL to PN. PdxI, which belongs to the AKR superfamily, exhibits weak homology to the known PL reductases. Our data showed that PdxI is also responsible for the accumulation of PN in the medium over time when *E. coli* is grown in the presence of PL. There are proteins homologous to PdxI in a wide range of bacteria and plants, suggesting that PL reductase in the B_6 salvage pathway is widely distributed in nature, despite being absent in a close relative of *E. coli*, *S. enterica*.

We updated the vitamin B_6 salvage pathway of *E. coli* taking account into the data described above and some results obtained with mutant strains that have single or multiple mutations in the enzyme(s) of the B_6 salvage pathway. The results are summarized in Table 2 and Fig. 1. As described, *E. coli* synthesizes PLP from exogenous PL using a detoured pathway (PL→PN→PNP→PLP) involving PdxI, PdxK, and PdxH rather than the direct pathway involving PdxK/PdxY kinases (PL→PLP). This mechanism was further supported by results showing the *pdxJ pdxK* mutant had poor growth in the

TABLE 2 Growth of *E. coli* strains that have single or multiple mutations in B_6 salvage enzymes

Genotype	Growth with[a]:		
	PL	PN	PM
pdxJ	+	+	+
pdxH	±	−	+
pdxJ pdxH	±	−	+
pdxJ pdxK	±	−	−
pdxJ pdxH pdxI	+	−	+
pdxJ pdxK pdxI	+	−	−

[a]+, grew well; ±, poor growth (final OD was less than 0.2 after 16 h of cultivation); −, no growth.

TABLE 3 Strains, plasmids, and primers

Strain, plasmid, or primer	Description or sequence (5′→3′)	Source or reference
E. coli strains		
BW25113	Wild type	Laboratory collection
DM16769	*pdxI*::Cm	This study
DM16696	*pdxH*::Kan	This study
DM16698	*pdxH*	This study
DM16027	*pdxJ*::*kan* (Keio collection, JW2548-KC)	33
DM16037	*pdxJ*	This study
DM16713	*pdxJ pdxH*::Kan	This study
DM16847	*pdxH*::Kan *pdxI*::Cm	This study
DM16865	*pdxH*::*kan pdxI pdxJ*	This study
AG1/pCA24N-pdxI	ASKA clone, JW1403-AM	32
Plasmid pCA24N-pdxI	N-terminal His-tagged PdxI expression (ASKA JW1403-AM)	32
Primers		
pdxH-H1	ATGTCTGATAACGACGAATTGCAGCAAATCGCGCATCTG CGCCGTGAATGTGTAGGCTGGAGCTGCTTCG	
pdxH-H2	TCAGGGTGCAAGACGATCAATCTTCCACGCATCATTTTC ACGCTGGTCATATGAATATCCTCCTTAG	
pdxI-H1	ATGAGCAGCAATACATTTACTCTCGGTACAAAATCCGTT AACCGTCTTGTGTAGGCTGGAGCTGCTTCG	
pdxI-H2	TTATTCTCGCGAAATACCATCCAACGTAGA CAACACTTCCTCAGAAAGATCATATGAATATCCTCCTTAG	
pdxH-check-fw	CGCATCGTCTTGAATAACTGTCAG	
pdxH-check-rv	CACCTTTGCCGGTACACGACTTTTC	
pdxI-check-fw	GCAACTCATCCAGTAATCTTGTTTACACC	
pdxI-check-rv	GTAAACGTATCCAGCCGCAATTCC	

presence of low levels of exogenous PL (0.1 μM), but the *pdxJ pdxK pdxI* triple mutant did not (Fig. 4B and C) (Table 2).

MATERIALS AND METHODS

Bacterial strains and media. All strains used in this study are derivatives of *Escherichia coli* BW25113 and are listed with their genotypes in Table 3. M9 medium supplemented with 0.2% glucose as the sole carbon source was used as the minimal medium for *E. coli* strains. Luria-Bertani (LB) broth was used for preculturing. Strains of *S. enterica* were grown in NCE medium. Agar (1.5%) was added for solid medium. When necessary, pyridoxal, pyridoxine, or pyridoxamine was added at designated concentrations. Antibiotics were added to the medium in a rich and minimal medium at the following final concentrations: ampicillin, 100 and 20 μg/ml; kanamycin, 50 and 10 μg/ml. Growth analyses in liquid medium were performed in a glass test tube using Taitec OD monitor C&T apparatus (for *E. coli* strains) or in 96-well microtiter plates in a BioTek ELx808 plate reader (for *S. enterica* strains). Media were incubated at 37°C with shaking, and growth was monitored every 30 min.

Deletion mutants of *pdxH* (DM16696) and *pdxI* (DM16769) were constructed with lambda Red recombineering as described previously using *E. coli* BW25113 as the parental strain (30). A kanamycin resistance cassette with a 40-bp homology region of the *pdxH* sequence was amplified by PCR with primers pdxH-H1 and pdxH-H2 and plasmid pKD4. A chloramphenicol resistance cassette flanked by 40-bp *pdxI* sequences was amplified by PCR with primers pdxI-H1 and pdxI-H2 and plasmid pKD3 as a template. These PCR products were purified from agarose gels and electroporated into the *E. coli* cells. Transformants were selected on an LB plate containing kanamycin (50 μg/ml) or chloramphenicol (30 μg/ml). Insertion of the antibiotic resistance gene at the *pdxH* or *pdxI* locus was confirmed by PCR using primer pair pdxH-check-fw/pdxH-check-rv or pdxI-check-fw/pdxI-check-rv. The antibiotic cassette was removed with pCP20 plasmid. When required, the *pdxJ*::Km, *pdxK*::Km, *pdxH*::Km, or *pdxI*::Cm mutations were transferred into the desired strain by P1 transduction as described previously (31).

Purification of PdxI. An *E. coli* AG1/pCA24N-PdxI strain (JW1403-AM [32]) was grown overnight in LB medium supplemented with chloramphenicol (30 μg/ml). Two milliliters of the preculture was inoculated into 200 ml of LB medium containing chloramphenicol and grown at 37°C with shaking to an optical density at 650 nm (OD_{650}) of 0.5. Isopropyl-β-D-thiogalactopyranoside (IPTG; 0.1 mM) was added to the medium, and cells were incubated for 4 h prior to harvesting by centrifugation (6,000 $\times$ *g*, 5 min, room temperature [RT]). The cell pellet was resuspended in a binding buffer containing 50 mM sodium phosphate (NaPB), 500 mM NaCl, 20 mM imidazole, pH 7.4, with DNase (0.025 mg/ml), lysozyme (1 mg/ml), and phenylmethylsulfonyl fluoride (0.1 mg/ml) and disrupted with a high-pressure cell disruption device, Constant Systems Limited One Shot (United Kingdom) at 20,000 lb/in², and cell lysate was cleared by centrifugation at 48,000 $\times$ *g* for 30 min at 4°C. The cell extract was passed through a 0.45-μm filter membrane and applied to a HisTrap HP Ni-Sepharose column (GE Healthcare) preequilibrated with the binding buffer. The column was washed with 5 column volumes of the binding buffer and an elution

buffer (20 mM NaPB, 500 mM NaCl, 500 mM imidazole, pH 7.4). Fractions containing PdxI, where the purified PdxI existed as a single band of ~31 kDa by SDS-PAGE, were combined and concentrated with a centrifugal filter device. The buffer was replaced with a buffer consisting of 20 mM NaPB and 10% glycerol (pH 7.4) with a PD10 desalting column (GE Healthcare), flash-frozen in liquid nitrogen, and stored at −80°C until use.

Enzyme assay. PL reductase activity catalyzed by PdxI was determined as described previously with slight modification (22, 24). Briefly, purified His-tagged PdxI (0.26 μg) was incubated in a reaction mixture containing 50 mM NaPB, 0.3 mM NADPH, and various concentrations (0, 0.025, 0.05, 0.1, 0.25, and 0.5 mM) of PL at 37°C. The reaction was started by the addition of purified PdxI. The decrease of NADPH absorption at 340 nm in a 1.0-ml reaction mixture was monitored over 5 min at 37°C. Control experiments were also performed in the absence of enzyme, PL, or NADPH.

B_6 vitamer analysis. B_6 vitamers were extracted from the cells with 10 volumes (vol/wt) of 0.9 M $HClO_4$ containing 50 μM deoxypyridoxine as an internal standard (100 μl of the $HClO_4$ solution for 10 mg [wet weight] *E. coli* cells). The suspension was vortex mixed and incubated on ice for 15 min, and 5 volumes (vol/wt) of 0.9 M K_2CO_3 solution (50 μl for 10 mg *E. coli* cells) was added. The mixture was centrifuged, and the resultant supernatant was diluted three times with water and used for the HPLC analysis (25 μl). The culture medium was deproteinized with $HClO_4$ (a final concentration of 0.9 M), neutralized by K_2CO_3, and clarified by centrifugation. The B_6 vitamers were separated with an octadecylsilyl (ODS) column (Cosmosil AR-II; Nacalai Tesque) (250 mm by 4.6 mm, 5-μm particle size) using a gradient program as described previously (17, 18). The flow rate was 1.0 ml per min, and the excitation and emission wavelengths were 328 nm and 393 nm, respectively.

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