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RESEARCH ARTICLE

Genetic Analysis Using Vitamin B_6 Antagonist 4-Deoxypyridoxine Uncovers a Connection between Pyridoxal 5′-Phosphate and Coenzyme A Metabolism in *Salmonella enterica*

Huong N. Vu,[a] Diana M. Downs[a]

[a]Department of Microbiology, University of Georgia, Athens, Georgia, USA

ABSTRACT Pyridoxal 5′-phosphate (PLP) is an essential cofactor for organisms in all three domains of life. Despite the central role of PLP, many aspects of vitamin B_6 metabolism, including its integration with other biological pathways, are not fully understood. In this study, we examined the metabolic perturbations caused by the vitamin B_6 antagonist 4-deoxypyridoxine (dPN) in a *ptsJ* mutant of *Salmonella enterica* serovar Typhimurium LT2. Our data suggest that PdxK (pyridoxal/pyridoxine/pyridoxamine kinase [EC 2.7.1.35]) phosphorylates dPN to 4-deoxypyridoxine 5′-phosphate (dPNP), which in turn can compromise the *de novo* biosynthesis of PLP. The data are consistent with the hypothesis that accumulated dPNP inhibits GlyA (serine hydroxymethyltransferase [EC 2.1.2.1]) and/or GcvP (glycine decarboxylase [EC 1.4.4.2]), two PLP-dependent enzymes involved in the generation of one-carbon units. Our data suggest that this inhibition leads to reduced flux to coenzyme A (CoA) precursors and subsequently decreased synthesis of CoA and thiamine. This study uncovers a link between vitamin B_6 metabolism and the biosynthesis of CoA and thiamine, highlighting the integration of biochemical pathways in microbes.

IMPORTANCE PLP is a ubiquitous cofactor required by enzymes in diverse metabolic networks. The data presented here expand our understanding of the toxic effects of dPN, a vitamin B_6 antagonist that is often used to mimic vitamin B_6 deficiency and to study PLP-dependent enzyme kinetics. In addition to *de novo* PLP biosynthesis, we define a metabolic connection between vitamin B_6 metabolism and synthesis of thiamine and CoA. This work provides a foundation for the use of dPN to study vitamin B_6 metabolism in other organisms.

KEYWORDS pyridoxal 5′-phosphate, vitamin B_6, 4-deoxypyridoxine, PtsJ, thiamine, CoA, coenzyme A, PLP, *ptsJ*

Pyridoxal 5′-phosphate (PLP) is the biologically active form of vitamin B_6 and a required cofactor in the metabolism of all organisms. Due to its unique chemical properties, PLP can facilitate diverse enzymatic reactions, including transamination, elimination, decarboxylation, and racemization (1). PLP-dependent enzymes, which are classified into seven fold types based on structural similarities (2), account for ~4% of all activities described by the Enzyme Commission (3). Most PLP-dependent enzymes, with the exception of glycogen phosphorylases, are associated with metabolic reactions that involve amino substrates (3).

In nature, PLP is synthesized *de novo* via one of two biosynthetic routes (Fig. 1). The deoxyxylulose 5-phosphate (DXP)-dependent pathway is found primarily in gammaproteobacteria such as *Salmonella enterica* and *Escherichia coli*, while the DXP-independent pathway is widespread among other bacteria, fungi, and archaea (4, 5). Alternatively, PLP can be acquired from other B_6 vitamers using the salvage pathway, as found in humans and animals (4, 5) (Fig. 1). Due to the reactivity of this cofactor and the prevalence

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Address correspondence to Diana M. Downs, dmdowns@uga.edu.

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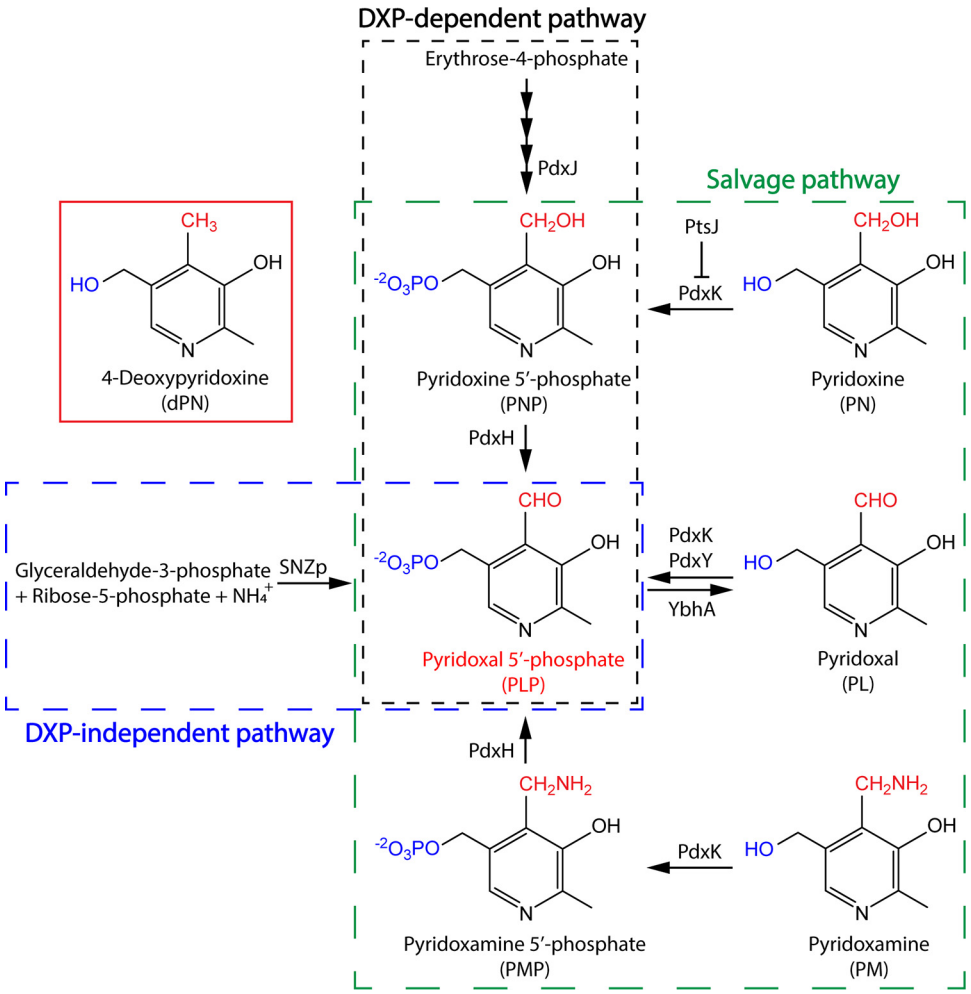

FIG 1 PLP biosynthesis and salvage. In *S. enterica*, PLP can be synthesized *de novo* via the DXP-dependent pathway (black box) or salvaged from other B_6 vitamers (green box). Other organisms, such as *Saccharomyces cerevisiae*, use the DXP-independent pathway (blue box) to synthesize PLP in addition to salvage. Animals, including humans, lack the biosynthetic pathway and rely exclusively on salvage to acquire this cofactor. Relevant enzymes involved in biosynthesis and salvage of PLP are shown next to the arrows, each of which represents a biochemical reaction catalyzed by the corresponding enzyme.

of PLP-dependent enzymes in metabolic networks, perturbations in PLP pools can inhibit both PLP-independent and PLP-dependent enzymes, causing adverse metabolic consequences (6–10). Despite the importance of this cofactor, the mechanism (or mechanisms) used by cells to control PLP homeostasis is not fully understood.

One approach to study vitamin B_6 metabolism involves the use of analogs, i.e., compounds that are structurally similar to B_6 vitamers but lack the biochemical properties to aid enzyme catalysis. In particular, 4-deoxypyridoxine (dPN) (Fig. 1) and its phosphorylated derivative 4-deoxypyridoxine 5′-phosphate (dPNP) have been used as potent vitamin B_6 antagonists in research for decades. Early studies in bacteria, fungi, plants, and animals focused on the inhibitory effects of dPN on growth, morphology, and the metabolism of other vitamins (11). Later work showed that dPN and dPNP could competitively inhibit the *in vitro* activity of the human pyridoxal (PL)/pyridoxine (PN)/pyridoxamine (PM) kinase (PdxK) (EC 2.7.1.35) (12) and pyridoxine 5′-phosphate (PNP)/pyridoxamine 5′-phosphate (PMP) oxidase (PdxH) (EC 1.4.3.5) (13), which are conserved enzymes essential for the salvage of B_6 vitamers (Fig. 1). This study was initiated to understand the mechanism of dPN toxicity in *S. enterica* serovar Typhimurium LT2 (referred to here as *S. enterica*) using phenotypic and metabolite analyses. Our results uncover a link between vitamin B_6 metabolism and the biosynthesis of thiamine and

coenzyme A (CoA), highlighting the complexity of metabolic networks and the primary consequences of perturbed pools of B_6 vitamers.

RESULTS AND DISCUSSION

Sensitivity of a *ptsJ* mutant to dPN is dependent on PdxK. While growth of an *S. enterica* wild-type strain in minimal no-carbon E (NCE) glycerol was not affected by 10 μM dPN, the presence of this antagonist inhibited growth if *pdxK* was overexpressed in *trans* (data not shown). Consistently, a strain lacking PtsJ, a MocR-type transcriptional repressor that regulates expression of *pdxK* (14), was sensitive to dPN in minimal NCE glucose in a dose-dependent manner (Fig. 2a). A null mutation in *pdxK* (Fig. 2a) or expression of *ptsJ* in *trans* (Fig. 2b) overcame the growth defect caused by dPN of the *ptsJ* mutant. These data showed the importance of PtsJ and PdxK in modulating dPN sensitivity.

Unphosphorylated B_6 vitamers are thought to enter cells via facilitated diffusion and subsequently be trapped by phosphorylation (15–17). In addition, the human homolog of PdxK could use dPN as a substrate *in vitro* (18). Taken together, the data suggest that, to be toxic, dPN was phosphorylated to dPNP by PdxK. Consistent with this hypothesis, high-performance liquid chromatography (HPLC) analysis showed that a *ptsJ* strain grown in minimal glucose medium supplemented with dPN (0.5 μM) accumulated intracellular dPNP ($\sim$555 $\pm$ 42 pmol/optical density at 650 nm [OD_{650}] unit). No dPNP was detected in cells grown in the absence of dPN (Fig. 2c). When a mutation in *pdxK* was introduced in the *pstJ* background, the profiles of intracellular B_6 vitamers in the absence and presence of dPN were indistinguishable. In total, these data support the conclusion that dPNP is the molecule that exerts toxic effects on *S. enterica* and, by extension, other organisms that possess homologs of PdxK.

DXP-dependent biosynthesis of PLP is compromised in the presence of dPNP. dPNP inhibited both the *E. coli* and human homologs of PdxH by competing with the native substrates PNP and PMP *in vitro* (13, 19). If such inhibition were to occur *in vivo*, then the growth defect of the *S. enterica ptsJ* mutant in the presence of dPN could be due to PLP limitation resulting from decreased PdxH activity. Growth of the *ptsJ* mutant in the presence of dPN was restored to different degrees with supplementation with 1 μM PL, PN, or PM (Fig. 3a). However, it was unclear whether these vitamers rescued growth by competing with dPN for transport, or for PdxK as the substrates or by increasing intracellular PLP content.

To help distinguish between these possibilities, the DXP-dependent PLP biosynthesis was eliminated in the *ptsJ* mutant by deleting *pdxJ* (encoding a PNP synthase [EC 2.6.99.2]) and *pdxH.* As a replacement for the native synthetic route, *Saccharomyces cerevisiae SNZ3* (encoding a PLP synthase [EC 4.3.3.6]) was expressed in *trans.* The resulting strain synthesizes PLP from endogenous glyceraldehyde-3-phosphate, ribose-5-phosphate, and exogenous ammonium in the medium using Snz3p (20) (Fig. 1). Growth of *ptsJ* strains carrying different PLP biosynthetic pathways in the presence of dPN was examined in minimal NCE glycerol (Fig. 3b). Strains lacking *ptsJ* and utilizing either the native (DXP-dependent) or heterologous (Snz3p-dependent) pathway for PLP synthesis grew similarly in the absence of dPN. When dPN was added, growth inhibition of the *ptsJ* strain synthesizing PLP with Snz3p was significantly less severe than that of the *ptsJ* strain using the native pathway. These data are consistent with the hypothesis that dPNP compromises the DXP-dependent PLP synthesis, while the remaining growth defect suggests that PdxH is not the only target of dPNP toxicity.

dPNP impacts ThiC-dependent thiamine synthesis. Probing the mechanism of dPNP toxicity beyond the presumed inhibition of PdxH required that the concentration of dPN be reduced to allow growth. Nutritional experiments showed that, in the presence of low levels of dPN (0.5 μM), the *ptsJ* strain was sensitive to adenosine, which was rescued by thiamine addition (Fig. 4). The ability of thiamine to abolish the synergistic effect of adenosine and dPN was reminiscent of previous studies, in which adenosine sensitivity indicated compromised synthesis of the pyrimidine moiety of thiamine (4-amino-5-hydroxymethyl-2-methylpyrimidine [HMP]). In those cases, adenosine

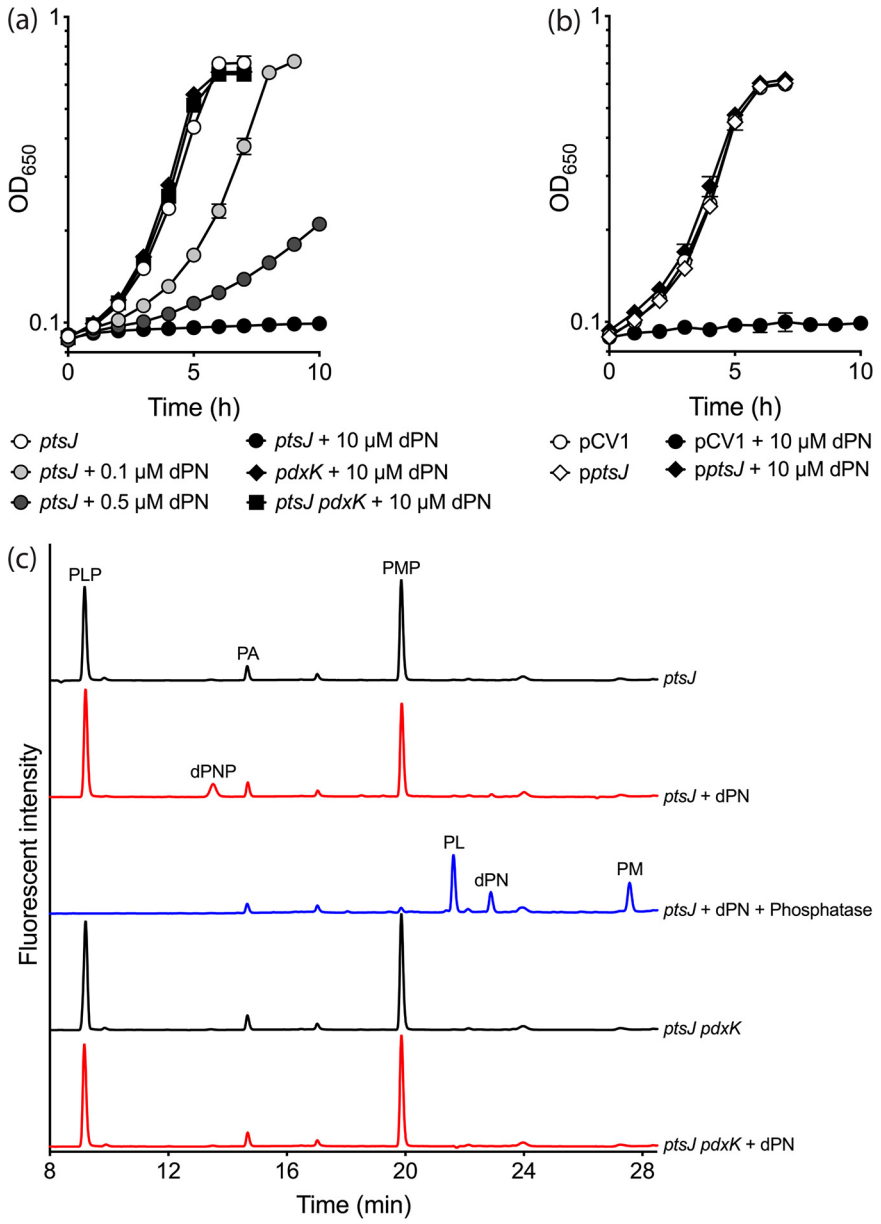

FIG 2 dPN sensitivity of a *ptsJ* mutant is PdxK-dependent. (a) *S. enterica ptsJ* (DM17239), *pdxK* (DM17238), and *ptsJ pdxK* (DM17168) were grown in minimal NCE glucose without (white symbols) or with addition of 0.1 μM (light gray symbols), 0.5 μM (dark gray symbols), or 10 μM (black symbols) dPN. (b) *ptsJ* strains carrying an empty vector control (pCV1) (DM17242) or a vector expressing *ptsJ* in *trans* (p*ptsJ*) (DM17243) were grown in minimal NCE glucose supplemented with 0.02% arabinose in the absence (white symbols) or presence (black symbols) of 10 μM dPN. (c) HPLC chromatogram of intracellular vitamin B_6 content of *ptsJ* (DM17239) and *ptsJ pdxK* (DM17168) strains grown in minimal NCE glucose with or without addition of 0.5 μM dPN. Representative data from at least two independent experiments with three biological replicates are shown. Error bars depict standard deviations from the mean values.

reduced flux toward 5-aminoimidazole ribotide (AIR) formation, which lowered HMP synthesis only if the efficiency of the AIR→4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate (HMP-P) reaction was diminished (21–24) (Fig. 5a). We hypothesized that the AIR→HMP-P conversion, catalyzed by ThiC (25, 26), was decreased in the presence of dPNP. In this scenario, the synergetic effects of adenosine and dPN would be due to reduced flux to AIR and reduced conversion of AIR to HMP-P, respectively. The effect of dPN on the efficiency of the AIR→HMP-P conversion was tested using a biosensor strain (DM7060) carrying a *ptsJ* mutation. The genotype of this strain allows the

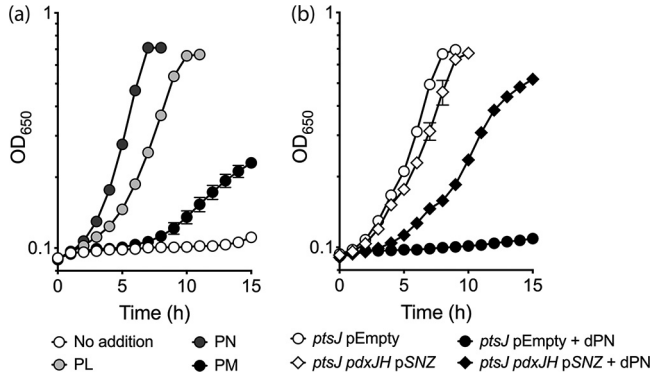

FIG 3 dPNP affects PLP synthesis via the DXP-dependent pathway. (a) The *S. enterica ptsJ* strain (DM17239) was grown in minimal NCE glucose supplemented with dPN (10 μM) in the absence (white symbols) or presence of PL (1 μM) (light gray symbols), PN (1 μM) (dark gray symbols), or PM (1 μM) (black symbols). (b) *S. enterica ptsJ* strains with the native DXP-dependent pathway intact (*ptsJ* pEmpty) (DM17244) or replaced with the *S. cerevisiae* DXP-independent pathway (*ptsJ pdxJH* p*SNZ*) (DM17246) were grown in minimal NCE glycerol supplemented with 0.02% arabinose in the absence (white symbols) or presence (black symbols) of dPN (10 μM). Representative data from at least two independent experiments with three biological replicates are shown. Error bars depict standard deviations from the mean values.

efficiency of the ThiC-dependent step to be assessed using 5-aminoimidazole riboside (AIRs) as a proxy (27, 28). This strain carries null alleles of *purG* and *purE*, which prevent flux to and from AIR, respectively, in *de novo* purine synthesis. In addition to purine auxotrophy, these mutations cause a thiamine requirement that can be rescued by supplementation of HMP or AIRs (Fig. 5a). A mutation in *stm4068* enables efficient utilization of exogenous AIRs by derepressing the kinase responsible for converting AIRs to AIR (29). Thus, the amount of AIRs that allows growth in this background is a reflection of the efficiency of the AIR→HMP-P conversion by ThiC.

The AIRs requirement of the *purGE stm4068 ptsJ* strain was assessed with soft agar overlay on minimal NCE glucose medium supplemented with adenine as a source for purines (Fig. 5b and c). In the absence of dPN, the mutant grew with addition of AIRs, HMP, or thiamine (Fig. 5b), as expected. When dPN (0.1 μM) was added to the medium, overall growth was not as robust. Nonetheless, it was still clear that growth was not supported by the amount of AIRs that allowed growth in the absence of dPN (Fig. 5c). These results indicated that the *ptsJ* mutant required more AIRs to grow in the presence of dPN, supporting the conclusion that the efficiency of AIR→HMP-P conversion by ThiC was compromised in the presence of dPNP.

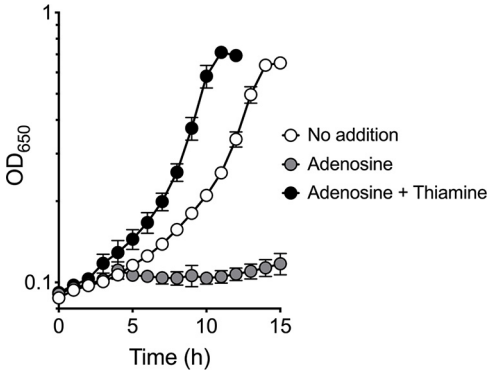

FIG 4 Adenosine exacerbates dPN sensitivity. The *S. enterica ptsJ* strain (DM17239) was grown in minimal NCE glucose supplemented with dPN (0.5 μM) in the absence (white symbols) or presence of adenosine (1 mM) (gray symbols) and thiamine (0.1 μM) (black symbols). Representative data from two independent experiments with three biological replicates are shown. Error bars depict standard deviations from the means.

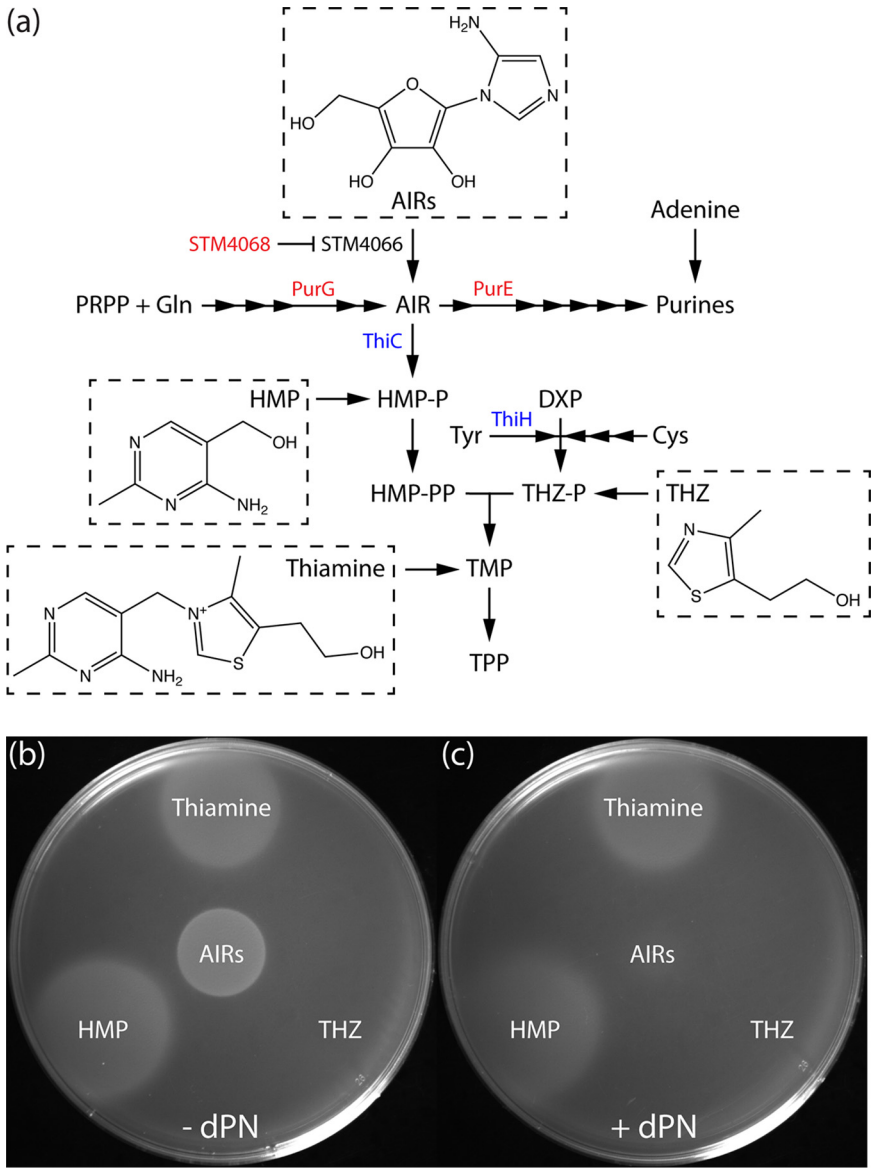

FIG 5 dPN increases the AIRs requirement for ThiC-dependent thiamine synthesis. (a) Thiamine and purine biosynthesis and salvage in *S. enterica*. Each arrow represents a biochemical reaction, and relevant enzymes are indicated next to the arrows. The DM7060 background carries lesions in genes that encode proteins in red. Blue indicates radical AdoMet enzymes that utilize iron-sulfur clusters. Compounds accompanied by chemical structures were used in subsequent feeding experiments. (b and c) Molten soft agar (0.7% [wt/vol]) inoculated with *purGE stm4068 ptsJ* culture (DM17274) was overlaid on minimal NCE glucose containing 0.4 mM adenine in the absence (b) or presence (c) of 0.1 μM dPN. Other supplementation was added by spotting 1 μL of AIRs (~300 mM), thiamine (0.1 mM), HMP (0.1 mM), and THZ (0.1 mM) on top of the solidified soft agar layer. Representative growth from three independent experiments is shown. PRPP, phosphoribosyl pyrophosphate; HMP-PP, 4-amino-5-hydroxymethyl-2-methylpyrimidine diphosphate; THZ-P, 4-methyl-5-(2-hydroxyethyl)-thiazole phosphate; TMP, thiamine monophosphate; TPP, thiamine pyrophosphate; Gln, L-glutamine; Tyr, L-tyrosine; Cys, L-cysteine.

dPNP inhibits the pantoate branch of CoA biosynthesis. Defects in several metabolic processes affect ThiC activity *in vivo*, including iron-sulfur cluster metabolism (28), synthesis of *S*-adenosylmethionine (AdoMet) from methionine (30), and intracellular CoA levels (31) (Fig. 6a). If dPNP caused a defect in iron-sulfur cluster synthesis/repair, both the HMP and thiazole (THZ) branches of thiamine synthesis would be expected to be compromised (28, 32–35) (Fig. 5a). However, only HMP-P synthesis was affected by dPNP (Fig. 5c). If the synthesis of AdoMet and/or CoA was compromised by dPNP, then methionine or pantothenate, respectively, should alter growth. The impact of these

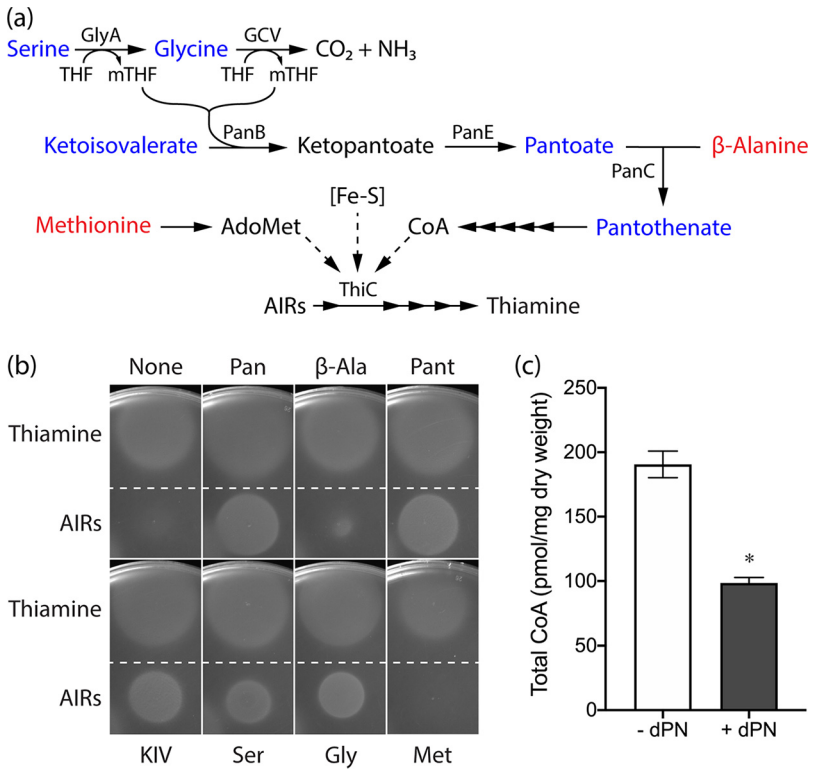

FIG 6 Precursors for CoA synthesis eliminate dPN sensitivity of a *ptsJ* mutant. (a) The integrated serine-glycine metabolic node, CoA, and thiamine synthesis in *S. enterica*. Blue indicates metabolites that mitigate the inhibition of dPN on ThiC-dependent thiamine synthesis, while those in red have little to no effect. Solid arrows indicate biochemical reactions in the pathways. Dashed arrows depict metabolic processes that affect ThiC activity. (b) The *purGE stm4068 ptsJ* strain (DM17274) was mixed with soft agar and overlaid onto minimal NCE glucose containing adenine (0.4 mM) and dPN (0.1 μM) without or with one of the following supplementations: pantothenate (0.1 mM), pantoate (0.1 mM), β-alanine (0.1 mM), KIV (0.1 mM), serine (2.5 mM), glycine (0.67 mM), or methionine (0.3 mM). The AIRs requirement was assessed by spotting 1 μL of AIRs (~300 mM) and thiamine (0.1 mM) as a control. (c) Total intracellular CoA level of a *ptsJ* mutant (DM17239) grown in minimal NCE glucose in the absence or presence of dPN (0.5 μM). *, $P < 0.0001$, as determined by two-tailed unpaired Student's *t* test. Representative data from two experiments, each with three biological replicates, are shown. Error bars depict standard deviations from the means. THF, tetrahydrofolate; Pan, pantothenate; β-Ala, β-alanine; Pant, pantoate; Ser, L-serine; Gly, L-glycine; Met, L-methionine.

additions on the AIRs requirement of the *purGE stm4068 ptsJ* strain was determined in the presence of dPN (Fig. 6b). Methionine had no effect on the AIRs requirement when dPN was present, making it unlikely that AdoMet synthesis was compromised. In contrast, growth in the presence of AIRs was significantly enhanced when either pantothenate or pantoate was added. These data implicated CoA biosynthesis, specifically the pantoate branch, as a target for the inhibitory effect of dPNP. Consistent with this hypothesis, a *ptsJ* mutant had a significant ~2-fold decrease in the total CoA pool when grown in medium supplemented with dPN (0.5 μM) (Fig. 6c). The weak stimulation by β-alanine was considered to be due to increased flux through PanC when the pantoate substrate level was decreased.

Addition of α-ketoisovalerate (KIV), serine, or glycine also decreased the AIRs requirement for growth in dPN (Fig. 6b). Serine and glycine are substrates in the generation of one-carbon units by GlyA (serine hydroxymethyltransferase [EC 2.1.2.1]) and the glycine cleavage (GCV) system, respectively (Fig. 6a). Both reactions produce 5,10-methylenetetrahydrofolate (mTHF) (36, 37), which is a cosubstrate for PanB (ketopantoate hydroxymethyltransferase [EC 2.1.2.11]) to convert KIV to ketopantoate (Fig. 6a). The PanB-dependent reaction was suggested to be the rate-limiting step for CoA synthesis (38). Notably, GlyA and GcvP (glycine decarboxylase [EC 1.4.4.2]) of the GCV system are PLP-dependent enzymes, which could be targeted by dPNP. There is precedent for damaged GlyA causing

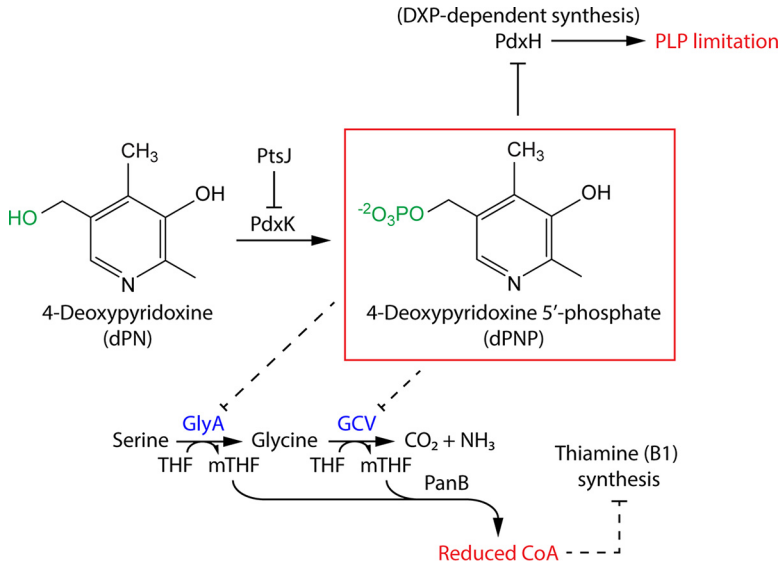

FIG 7 Working model regarding how dPN affects *S. enterica* metabolism. PdxK converts exogenous dPN to dPNP, which inhibits PLP biosynthesis via PdxH, leading to PLP limitation. In addition, dPNP inhibits the PLP-dependent enzyme GlyA and/or GcvP, decreasing flux through the pantoate branch of CoA synthesis. Reduced CoA levels indirectly dampen ThiC-dependent thiamine synthesis. THF, tetrahydrofolate.

a decrease in CoA levels in *S. enterica* (39). In addition, mitogen-induced GlyA activity measured in crude extracts was decreased when human leukocytes and T cells were cultured with dPN supplementation, and the activity was rescued by the addition of vitamin B_6 (40). Although the effect of dPNP on GcvP has not been reported, PNP, a B_6 vitamer with a structure similar to that of dPNP (Fig. 1), could inhibit the *in vitro* activity of *E. coli* and *Bacillus subtilis* GCV systems (41). In total, the data are consistent with a model in which dPNP inhibits GlyA and/or the GCV system by competing with PLP. Such inhibition would lead to a reduction in mTHF levels and decrease flux to CoA synthesis.

Working model for growth limitation by dPN. The cumulative effects of dPN toxicity, exacerbated by a *ptsJ* mutation, are depicted in Fig. 7. Our working model suggests that exogenous dPN is phosphorylated to dPNP by PdxK (Fig. 2). Intracellular dPNP then competes with PNP for binding to PdxH and thus compromises the DXP-dependent synthesis of PLP (Fig. 3). Additionally, dPNP inhibits the activity of the GlyA and/or GcvP PLP-dependent enzymes, which reduces the levels of mTHF. Finally, lower-than-optimal mTHF levels compromise the activity of PanB, reducing formation of pantothenate and decreasing flux to CoA (Fig. 6). Reduced CoA levels then indirectly affect the synthesis of the pyrimidine moiety of thiamine, resulting in overall lower thiamine formation (Fig. 4 and 5).

The conclusions made from phenotypic analyses are bolstered by considering the kinetic parameters of PdxH. Based on HPLC data, the intracellular concentration of dPNP was estimated to be ~4-fold and 350-fold higher than those of PMP and PNP, respectively, under conditions where growth was significantly impaired (data not shown). The binding affinity of dPNP for *E. coli* PdxH is similar to that of PMP and ~50-fold lower than that of PNP (19). Together, these considerations support the ability of dPNP to compete with PNP and/or PMP for PdxH and reduce its ability to generate PLP. Similar kinetic parameters with GlyA and GcvP have not been reported, and additional *in vitro* experiments are required to further support the genetic analyses.

It was somewhat surprising that PdxY was not involved in dPNP toxicity, since it is another PL kinase (EC 2.7.1.35) involved in B_6 salvage (42). PdxK and PdxY have promiscuous kinase activity with pyrimidine and pyridine derivatives, including the HMP moiety of thiamine and unphosphorylated B_6 vitamers (42–45). A potential explanation for the lack of a role for PdxY is that *S. enterica* evolved differential regulation of *pdxK* and

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
S. enterica strains		
DM17239	*ptsJ617*::*Km*	This study
DM17238	*pdxK682*::*Cm*	This study
DM17168	*ptsJ617*::*Km pdxK682*::*Cm*	This study
DM17274	*purG3111 purE3043 stm4068-6*::Tn*10*(d)Tc *ptsJ617*::*Km*	This study
DM16666	Wild-type/pCV1	This study
DM17210	Wild-type/pDM1643	This study
DM17242	*ptsJ617*::*Km*/pCV1	This study
DM17243	*ptsJ617*::*Km*/pDM1647	This study
DM17253	*ptsJ617*::*Km pdxK682*::*Cm*/pCV1	This study
DM17244	*ptsJ617*::*Km*/pBAD33-SD1	This study
DM17246	*pdxJ664 pdxH673 ptsJ617*::*Km*/pDM1594	This study
Plasmids		
pBAD33-SD1	Modified pBAD33 (Cm^r)	55
pCV1	Modified pBAD24 (Am^r)	50
pDM1594	pBAD33-SD1 expressing *S. cerevisiae SNZ3* (Cm^r)	55
pDM1643	pCV1 expressing *S. enterica pdxK* (Am^r)	This study
pDM1647	pCV1 expressing *S. enterica ptsJ* (Am^r)	This study
pBsPTA1	pTEV5 expressing *B. subtilis* 6×His-tagged PTA (Am^r)	Escalante-Semerena[a]

[a]Provided by Jorge C. Escalante-Semerena at the University of Georgia (Athens, GA).

pdxY. Strains lacking PtsJ, like those used here, led to an ~80-fold increase in expression of *pdxK*, while only a 2- to 3-fold increase was observed with *pdxY* (14). Alternatively, PdxY may not phosphorylate dPN with the efficiency that would make it relevant under the conditions tested.

This work did not address whether dPN/dPNP could be metabolized or detoxified in *S. enterica*. Rapid excretion and modification of dPN was proposed to be a detoxifying mechanism in humans and rats, in which dPN appeared to be converted to 4-deoxy-5-pyridoxic acid and deoxypyridoxine-3-(hydrogen sulfate), respectively (46). Isolation and characterization of *S. enterica* mutants that are more or less sensitive to dPN could address whether such a mechanism exists in bacteria.

In total, this study contributes to our understanding of the mechanism by which exogenous dPN inhibits growth in *S. enterica*. The data presented here provide a framework for probing the metabolic network structure involving vitamin B_6 in other organisms. Furthermore, use of dPN provides an additional means to disrupt PLP metabolism, which can be coupled with mutations that impact PLP-dependent enzymes and PLP homeostasis, such as *ridA* and *yggS,* respectively, to better understand additional metabolic aspects of this critical cofactor.

MATERIALS AND METHODS

Strains, plasmids, and primers. Strains and plasmids are presented in Table 1. Primers used for strain and plasmid construction are listed in Table 2. Strains are derivatives of *S. enterica* serovar Typhimurium LT2. *Escherichia coli* DH5α and BL21AI (Invitrogen, Carlsbad, CA) were used to propagate plasmids and purify recombinant protein, respectively. Phage λ Red recombineering (47) was adapted for *S. enterica* to generate gene deletions. Initial deletions were transduced into appropriate strain backgrounds using phage P22 *HT105/1 int-201* (48) following published protocol (49) and confirmed by colony PCR.

Plasmid pDM1643 and pDM1647 were constructed by cloning *S. enterica pdxK* and *ptsJ*, respectively, into pCV1 (50) as described (51) and were validated using Sanger sequencing (Eton Bioscience, San Diego, CA). Plasmid pBsPTA1 was generated by cloning *pta* (encoding a phosphotransacetylase [PTA] [EC 2.3.1.8]) from *B. subtilis* 168 into pTEV5 (52) and was a gift from Jorge C. Escalante-Semerena at the University of Georgia (Athens, GA). Plasmids were electroporated into *S. enterica* and *E. coli* using a standard protocol.

Media and chemicals. Strains were grown at 37°C in nutrient broth (8 g/L Difco mix, 5 g/L NaCl) or minimal NCE medium supplemented with 1 mM $MgSO_4$, trace metals (53), and 11 mM glucose or 22 mM glycerol as the sole carbon source. Lysogeny broth (10 g/L Bacto tryptone, 5 g/L yeast extract, 5 g/L NaCl) and super broth (32 g/L Bacto tryptone, 20 g/L yeast extract, 5 g/L NaCl, 5 mM NaOH) were used to culture *E. coli* for protein purification. Agar was added to a final concentration of 1.5% (wt/vol) to obtain solid media. Additional supplements included dPN (0.1 to 10 μM), PL (1 μM), PN (1 μM), PM (1 μM), adenosine (1 mM), adenine (0.4 mM), pantothenate (0.1 mM), pantoate (0.1 mM), β-alanine

TABLE 2 Primers used in this study

Primer	Sequence (5′ to 3′)	Description
PR1383	GGAAAATTTTATGGGACAAGAGAGTGATATTCAGTCAGTGGTGTAGGCTGGAGCTGCTTC	Inactivation of *pdxK*
PR1384	CGATAACTCTTCATCGCGCCTCCCCTGCCGGCGGCAGAATCATATGAATATCCTCCTTAGTTCCTATTCC	Inactivation of *pdxK*
PR1461	ATTAAAGGCAGTCCGTCTCCCGTAGCGCTGGAAGGCGGCGTGTAGGCTGGAGCTGCTTC	Inactivation of *ptsJ*
PR1462	TCCCTCACGTACCAGCCAGCCGGATTTTGCCAACGTAAACATATGAATATCCTCCTTAGTTCCTATTCC	Inactivation of *ptsJ*
PR1318	NNGCTCTTCNTTCATGGGACAAGAGAGTGATATTCAGTCAGTG	Construction of pDM1643
PR1319	NNGCTCTTCNTTATCATCGCGCCTCCCCTG	Construction of pDM1643
PR1449	NNGCTCTTCNTTCATGATCGACGGAAAAACCGCTAACGAAATTTTTGACAG	Construction of pDM1647
PR1450	NNGCTCTTCNTTATTAGCGATTTAGCGCCTGATGGATATCAGCC	Construction of pDM1647

(0.1 mM), KIV (0.1 mM), serine (2.5 mM), glycine (0.67 mM), methionine (0.3 mM), or thiamine (0.1 μM) as indicated in the text. When needed, antibiotics were added as followed: ampicillin (Am), 150 μg/mL in rich medium and 15 μg/mL in minimal medium; chloramphenicol (Cm), 20 μg/mL in rich medium and 5 μg/mL in minimal medium; kanamycin (Km), 50 μg/mL.

Unless otherwise stated, all chemicals were purchased from MilliporeSigma (formerly Sigma-Aldrich, St. Louis, MO). Crude dPNP was obtained from Cayman Chemical (Ann Arbor, MI). PNP was synthesized from PLP by reduction with $NaBH_4$ (45). PMP was synthesized by dissolving PLP in concentrated NH_4OH, which was subsequently reduced by the addition of $NaBH_4$ (54). Restriction enzymes and *Taq* polymerase were purchased from New England BioLabs (Ipswich, MA). Primers were synthesized by Eton Bioscience (San Diego, CA).

Growth analysis. Strains were precultured in 2 mL nutrient broth at 37°C for 6 to 8 h in an Innova 43 shaker (New Brunswick Scientific, Edison, NJ) at 250 rpm. Cells were washed with an equal volume of 0.85% NaCl and inoculated (2.5% [vol/vol]) into minimal NCE medium containing appropriate carbon sources and supplements. Growth was monitored in a 96-well plate by following the OD_{650} over time using a Elx808 plate reader (BioTek Instruments, Winooski, VT). Data were visualized using GraphPad Prism v8.4.3 (GraphPad Software, La Jolla, CA).

HPLC analysis of B_6 vitamers. Intracellular vitamin B_6 profiles of *ptsJ* (DM17239) and *ptsJ pdxk* (DM17168) strains grown in minimal NCE glucose without or with addition of 0.5 μM dPN were determined following a described protocol (55) except that dPN was replaced by 4-pyridoxic acid (PA) as an internal standard. The identity of the dPNP peak was confirmed by coinjection of crude dPNP with the samples and by treatment with wheat germ acid phosphatase (55). The level of internal dPNP accumulation was inferred by quantifying the concentration of dPN in phosphatase-treated samples.

Assessment of AIRs requirement. *S. enterica purGE stm4068 ptsJ* (DM17274) was precultured in 2 mL nutrient broth and washed as described for growth analysis. Three milliliters of molten soft agar (0.7% [wt/vol]) seeded with 100 μL washed cells was overlaid onto minimal NCE glucose containing adenine (0.4 mM), without or with the addition of dPN (0.1 μM), pantothenate (0.1 mM), pantoate (0.1 mM), β-alanine (0.1 mM), KIV (0.1 mM), serine (2.5 mM), glycine (0.67 mM), or methionine (0.3 mM). One microliter of ~300 mM AIRs, 0.1 mM thiamine, 0.1 mM HMP, and 0.1 mM THZ was subsequently spotted on top of the solidified agar layer. After overnight incubation at 37°C, plates were photographed using a FOTO/Analyst FX workstation (Fotodyne, Hartland, WI).

Purification and activity assay of *B. subtilis* PTA. The *E. coli* BL21Al strain carrying pBsPTA1 was precultured in 10 mL lysogeny broth in duplicate at 37°C in an Innova 43 shaker (New Brunswick Scientific) at 250 rpm. Overnight cultures were inoculated into two Fernbach flasks, each of which contained 1.5 L of super broth. Growth was resumed at 37°C in an Innova 44 shaker (New Brunswick Scientific) at 180 rpm until mid-log phase (OD_{650} of ~0.6), as determined by a Spectronic 20D+ instrument (Thermo Fisher Scientific, Waltham, MA). After the addition of arabinose (0.02% [wt/vol]) and isopropyl-β-D-thiogalactopyranoside (0.5 mM) to induce expression of *pta*, the temperature was shifted to 30°C. Cells were harvested (6,000 $\times$ *g* for 15 min at 4°C) after 20 h and stored at −80°C until purification.

Protein purification and dialysis steps were performed at 4°C. The frozen cell pellet was resuspended (3 mL/g wet weight) in buffer A (50 mM HEPES, 500 mM NaCl, 20 mM imidazole [pH 7.5]) containing lysozyme (1 mg/mL), DNase (0.14 mg/mL), and phenylmethylsulfonyl fluoride (PMSF) (1 mM) and was lysed at 18,000 lb/in^2 using a Constant Systems Limited One Shot cell disruptor (Northants, UK). The cell lysate was clarified (45,000 $\times$ *g* for 45 min at 4°C), filtered (0.45-μm polyvinylidene difluoride [PVDF]), and loaded onto a preequilibrated 5-mL HisTrap HP Ni-Sepharose column (GE Healthcare, Chicago, IL) using an NGC Quest 10 chromatography system (Bio-Rad, Hercules, CA). The loaded column was washed and eluted with 10 column volumes (CVs) of buffer A, 6 CVs of 4% buffer B (50 mM HEPES, 500 mM NaCl, 500 mM imidazole [pH 7.5]), and a 10-CV gradient of 4 to 100% buffer B at a flow rate of 2 mL/min. Fractions containing PTA were visualized on a 14% SDS-PAGE gel and pooled. Protein was concentrated using an Amicon Ultra-15 30K centrifugal filter unit (MilliporeSigma), dialyzed overnight against buffer C (50 mM HEPES, 150 mM NaCl, 10% glycerol [pH 7.5]), flash-frozen with liquid nitrogen, and stored at −80°C until assay. The protein concentration was determined spectrophotometrically using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific), from the calculated molecular mass and extinction coefficient (ExPASy).

Enzyme activity was assayed using a previously described protocol (56) adapted for 96-well plates. Briefly, 0 to 10 ng of PTA was added to a 300-μL reaction mixture containing 100 mM Tris-HCl (pH 7.4), 1.6 mM glutathione, 0.4 mM CoA, 7.2 mM acetyl phosphate, and 13.3 mM $(NH_4)_2SO_4$ to initiate the

reaction. Acetyl-CoA formation was monitored at 233 nm (ε_{233} = 4.44 mM^{-1} cm^{-1}) in a 96-well quartz plate using a SpectraMax 398 Plus plate reader (Molecular Devices, Sunnyvale, CA) and normalized to a 1-cm pathlength. One unit of PTA converts 1 μmol of CoA to acetyl-CoA per minute at 25°C (pH 7.4) using acetyl phosphate as a substrate.

CoA quantification. Total CoA was determined as described (57, 58) with modifications. The *S. enterica ptsJ* strain (DM17239) was precultured in 10 mL nutrient broth in triplicate overnight. Cells were washed with an equal volume of 0.85% NaCl and inoculated (2% [vol/vol]) into Klett flasks containing 200 mL minimal NCE glucose without or with 0.5 μM dPN supplementation. After reaching an OD_{650} of 0.7 to 0.8, cells were harvested (8,000 $\times$ *g* for 15 min at 4°C), washed with 5 mL cold phosphate-buffered saline (PBS), and stored at −80°C until analysis.

Frozen cell pellets were resuspended in cold PBS and lysed by the addition of formic acid to a final concentration of 0.3 N. After a 30-min incubation on ice with occasional agitation, cell debris was pelleted (17,000 $\times$ *g* for 10 min at 4°C), and the lysate was neutralized with NH_4OH (pH ~6.5 to 7.5). Dithiothreitol (0.7% [vol/vol]) was added to aliquots of neutralized lysate to reductively cleave CoA thioesters. The assay mixture (120 μL) contained 250 mM Tris-HCl (pH 7.2), 50 mM KCl, 15 mM malate, 6 mM acetyl phosphate, 1 mM NAD^+, 0.4 U porcine citrate synthase, 2 U porcine malate dehydrogenase, 40 μL treated extract, and 0.8 U *B. subtilis* PTA, which was added last to initiate the reaction. NADH formation was monitored at 340 nm in a 96-well quartz plate and normalized to a 1-cm pathlength using a SpectraMax 398 Plus plate reader (Molecular Devices). The CoA level for each biological sample was measured with four technical replicates and normalized to dry cell weight. A 7-point standard curve was generated with the addition of 0 to 1.2 nmol CoA and was used as reference for CoA quantification.

Data availability. All relevant data are included in the content of the manuscript.

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