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Isolation of a *pdxJ* Point Mutation That Bypasses the Requirement for the PdxH Oxidase in Pyridoxal 5′-Phosphate Coenzyme Biosynthesis in *Escherichia coli* K-12

TSZ-KWONG MAN, GENSHI ZHAO, AND MALCOLM E. WINKLER*

Department of Microbiology and Molecular Genetics, University of Texas Houston Medical School, Houston, Texas 77030-1501

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We isolated 26 suppressor mutations that allowed growth of a Δ*pdxH*::Ω null mutant in the absence of pyridoxal. Each suppressor mapped to *pdxJ*, and the eight suppressors sequenced contained the same glycine-to-serine change in the PdxJ polypeptide. This bypass suppression suggests that PdxJ may participate in formation of the pyridine ring of pyridoxine 5′-phosphate.

The native pyridoxine 5′-phosphate (PNP)/pyridoxamine 5′-phosphate (PMP) oxidase encoded by the *pdxH* gene of *Escherichia coli* acts as a classical, monofunctional, dimeric flavoprotein oxidase under aerobic assay conditions (40). This enzyme uses molecular oxygen as an electron acceptor to oxidize the C-4′ alcohol or amine group of PNP or PMP, respectively, to form the active coenzyme pyridoxal 5′-phosphate (PLP) and hydrogen peroxide (H_2O_2) (Fig. 1) (21, 40). The *E. coli* PdxH PNP/PMP oxidase plays roles in both the main de novo pathway of PLP biosynthesis (Fig. 1) and the salvage pathway that interconverts the six B_6 vitamers, pyridoxal (PL), pyridoxamine (PM), pyridoxine (PN; vitamin B_6), PLP, PMP, and PNP (Fig. 1) (9, 18, 19, 26, 31, 40). An important question about PLP biosynthesis is how PNP oxidation to PLP occurs in anaerobically growing *E. coli* cells in which the PNP/PMP oxidase presumably does not function. There are several precedents for the induction of a second enzyme that replaces oxidase function in facultative anaerobes, such as *E. coli* (25, 34, 35). However, a functioning *pdxH* gene is required for de novo PLP biosynthesis by *E. coli* growing in the presence or absence of molecular oxygen (8, 18). One hypothesis that explains these growth properties is that there is an anaerobic oxidoreductase that still requires the *pdxH* gene product for function or expression.

To test this hypothesis, we constructed a stable *pdxH* null mutant (Fig. 2) and selected for suppressor mutations in anoxic *E. coli* cells. This Δ*pdxH*::Ω(Cm^r) mutation had the middle third of the *pdxH* coding region replaced by an omega (Ω) cassette that imparts chloramphenicol resistance (Cm^r) (Fig. 2) (22). The Δ*pdxH*::Ω(Cm^r) mutation was crossed into the bacterial chromosome (Table 1) (2, 32). The resulting mutant (TX2768) had the expected phenotype of growth on MM-glucose plates [(1 × E) minimal salts (7) containing 0.4% (wt/vol) glucose, 0.01 M $FeSO_4$, 1.5% (wt/vol) Bacto agar] supplemented with 1.0 μM PL, but not PN, since PNP/PMP oxidase is required for the conversion of PNP to PLP (8, 16, 18, 40). PCR analyses of genomic DNA (27) confirmed the location of the Δ*pdxH*::Ω(Cm^r) mutation, and crude extracts of TX2768 [Δ*pdxH*::Ω(Cm^r)] lacked detectable PNP/PMP oxidase activity and a PdxH protein band on Western immunoblots (data not shown) (40).

We selected for suppressors of the Δ*pdxH*::Ω(Cm^r) null mutation on plates containing MM-glucose medium without PL at 37°C in an anaerobic chamber. We retained 6 independent spontaneous suppressor mutants and 20 suppressor mutants (2 each from 10 independent cultures) isolated after mutagenesis with 2-aminopurine (2AP) (20). We mapped the 2AP-induced suppressor mutation from strain TX2773 in the following way. We prepared a genomic library of DNA from the suppressor mutant in vector pBR322 (4, 38). Transformants of the library that suppressed the Δ*pdxH*::Ω(Cm^r) mutation in TX2768 were selected on MM-glucose plates containing antibiotics (25.0 μg of ampicillin and 12.5 μg of chloramphenicol per ml) at 37°C in the absence of oxygen. Plasmid DNA (pTX366) from the suppressor hybridized to Kohara phage 434 (58 min) on an *E. coli* gene mapping membrane (Takara Biochemical Inc., Berkeley, Calif.) (14). The restriction map of recombinant suppressor plasmid pTX366 matched that at 58 min in the *E. coli* chromosome, which includes the *rnc-era-recO-pdxJ-dpj* superoperon (16, 24). *pdxJ*, which is partly cotranscribed with *recO* and partly transcribed from an internal promoter (P_{pdxJ}) at the end of *recO* (16), is required for de novo PLP biosynthesis (Fig. 1) (16, 24). The function of *pdxJ* is not exactly known, but indirect evidence suggested that it may mediate ring closure between 4-phospho-hydroxy-L-threonine (4PHT) and D-1-deoxyxylulose (DX) (or D-1-deoxyxylulose-5-phosphate [DXP]) (Fig. 1; see below also). Recombinant suppressor plasmid pTX366 complemented *pdxJ19* mutant NU927 (Table 1). A minimal [*recO*′(P_{pdxJ})-*pdxJ*] subclone (pTX379 [Table 1]) of recombinant suppressor plasmid pTX366 also complemented *pdxJ19* mutant NU927 and suppressed the Δ*pdxH*::Ω(Cm^r) mutation (data not shown). In contrast, the likely overexpression of *pdxJ*$^+$ from multicopy plasmid pNU199 did not suppress the Δ*pdxH*::Ω(Cm^r) mutation in TX2768. Thus, this suppressor mutation of the Δ*pdxH*::Ω(Cm^r) insertion-deletion appeared to be in *pdxJ*.

We tested the remaining 25 suppressors of the Δ*pdxH*::Ω(Cm^r) mutation for their linkage to *pdxJ*. Each of the 25 suppressors was crossed out at nearly 100% frequency by transduction with P1*vir* phage (20) grown on strain NU1428 [*recO*::miniMu*d*II

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, University of Texas Houston Medical School, 6431 Fannin, JFB 1.765, Houston, TX 77030-1501. Phone: (713) 794-1744, ext. 1526. Fax: (713) 794-1782. Electronic mail address: mwinkler@utmmg.med.uth.tmc.edu.

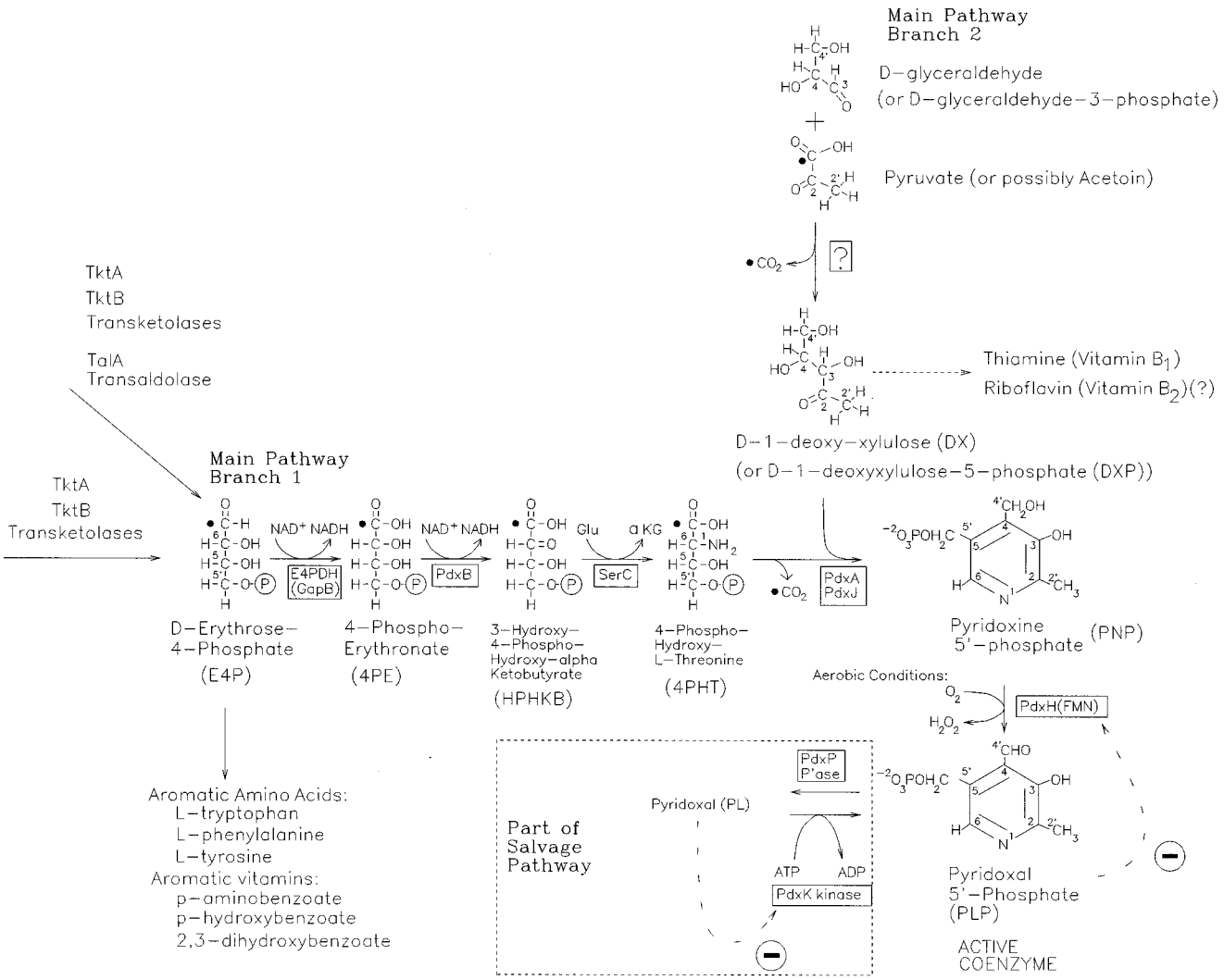

FIG. 1. Proposed pathway for the de novo biosynthesis of PLP coenzyme in *E. coli* K-12. The steps in branch 1 leading to 4PHT have been confirmed genetically and biochemically (10, 13, 33, 38, 39, 41). 4PHT and DX (or DXP) were shown to be in vivo precursors of PLP (10, 11, 13, 33). The steps leading to DX (or DXP) are not exactly known (36, 37), and DX (or DXP) is thought to be a precursor of thiamine and possibly riboflavin (5, 6, 29, 30). The postulated role of PdxJ and PdxA in pyridine ring closure was based solely on the growth properties of mutants (see text) (10, 17). The first B_6 vitamer synthesized de novo is likely PNP (41), which is then converted to PLP by PdxH PNP/PMP oxidase activity (8, 18, 21, 40). The PdxH PNP/PMP oxidase, PdxK PL/PM/PN kinase, and PdxP phosphatase(s) interconvert the six B_6 vitamers (PL, PM, PN, PLP, PMP, and PNP) in the salvage pathway (9, 28, 31, 40). Feedback inhibition of PdxH PNP/PMP oxidase by PLP (21, 40) and PdxK PL/PM/PN kinase by PL (31) is indicated by the circled minus signs. Numbering of carbon and nitrogen atoms refers to positions in PLP. Carbon atoms released in the pathway are marked with small black dots.

(Km^r) *pdxJ*$^+$] (Table 1). Thus, all of the Δ*pdxH*::Ω(Cm^r) suppressor mutations were closely linked to and possibly in the *pdxJ* gene. To test this hypothesis further, we sequenced the entire *pdxJ* gene of PCR-amplified genomic DNA (38) isolated from four independent spontaneous suppressor mutants and four independent 2AP-induced suppressor mutants, including the one used in the above mapping strategy. Each mutant contained a single G-to-A transition at nucleotide 2230 in *pdxJ*, using the numbering in reference 16. This G-to-A mutation caused a single amino acid change of glycine to serine at position 194 in the PdxJ polypeptide chain, designated PdxJ (G194S).

We characterized the physiological properties caused by the *pdxJ*(G194S) mutation to understand the mechanism by which it suppressed the *pdxH* null mutation. The *pdxJ*(G194S) mutation contained on the chromosome or on high-copy-number vector pUC18 did not restore detectable PNP or PMP oxidase enzyme activities to the *pdxH* null mutant (data not shown) (40). This result suggested that *pdxJ*(G194S) might be a bypass mutation that allowed the formation of the C-4′ aldehyde group of PLP by a new biosynthesis route (Fig. 1). Consistent with this interpretation, the *pdxJ*(G194S) mutation suppressed the Δ*pdxH*::Ω(Cm^r) mutation in cells growing aerobically or anaerobically as in the initial selection (data not shown).

Another indication that the *pdxJ*(G194S) mutation caused bypass synthesis of PLP came from the growth properties of *pdxA* (or *pdxB*) *pdxJ*(G194S) Δ*pdxH*::Ω(Cm^r) triple mutants (Table 1). The *pdxA* and *pdxB* mutations block de novo synthesis of PNP (Fig. 1); however, PN taken up from the culture medium is phosphorylated to PNP by PL/PM/PN kinase in the B_6 vitamer salvage pathway (9, 26, 31). If the *pdxJ*(G194S) suppressor mutation provided an alternate oxidase activity in the *pdxH* null mutant, then PNP formed from exogenously added PN should support growth of the triple mutants. Con-

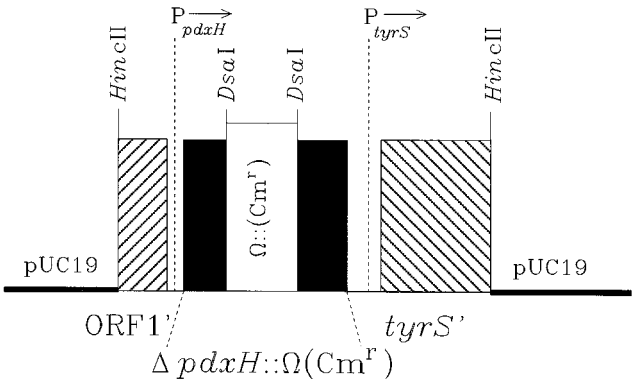

FIG. 2. Map of the Δ*pdxH*::Ω(Cm^r) deletion-insertion null mutation constructed in plasmid pTX363 (Table 1). Two small *Dsa*I restriction fragments (285 bp together) in the middle of *pdxH* (originally 656 bp) were replaced with a 3.5-kb Ω(Cm^r) cassette (Table 1). The locations of the partial fragments of upstream ORF1′ and downstream *tyrS*′ (hatched), the remaining flanking parts of *pdxH* (solid), and the P_{pdxH} and P_{tyrS} promoters are indicated. The Δ*pdxH*::Ω(Cm^r) null mutation was crossed into the chromosome of NU816 (2, 32) to form strain TX2768 (Table 1).

trary to this expectation, the triple mutants grew only on medium supplemented with PL (data not shown), which does not require oxidation to enter the salvage pathway (Fig. 1).

Complementation experiments showed that overexpression of wild-type PdxJ$^+$ protein interfered with suppression of the Δ*pdxH*::Ω(Cm^r) mutation by the mutant PdxJ(G194S) protein. In the mapping scheme described above, the *pdxJ*(G194S) mutation contained on medium-copy-number plasmid pBR322 suppressed the Δ*pdxH*::Ω(Cm^r) mutation in a *pdxJ*$^+$ strain. However, a minimal P_{pdxJ}-*pdxJ*$^+$ clone in pBR322 (pTX376) interfered with the suppression of the Δ*pdxH*::Ω(Cm^r) mutation by a chromosomal *pdxJ*(G194S) mutation (Fig. 3). This result suggested that PdxJ may exist as a multimer or that it interacts with another enzyme. Alternatively, overexpressed wild-type PdxJ$^+$ protein may compete with the PdxJ(G194S) mutant protein for a compound that the mutant protein uses in the ring closure reaction to produce PLP directly (see below). Finally, *pdxJ*(G194S) Δ*pdxH*::Ω(Cm^r) mutants appeared to grow slower and biphasically compared with their *pdxJ*$^+$ *pdxH*$^+$ parents (Fig. 3). Thus, the *pdxJ*(G194S) suppressor did not fully restore growth to the Δ*pdxH*::Ω(Cm^r) mutant, possibly because the amount of PLP was not sufficient or the bypass pathway siphoned off an intermediate from another pathway.

The role of PdxJ in PNP and PLP biosynthesis is largely unknown (9, 12, 16, 17). On the basis of the growth patterns of different *pdx* mutants on supplemented MM-glucose medium, PdxJ and PdxA were proposed to function after the synthesis of 4PHT, probably in the ring closure reaction between 4PHT and DX (or DXP) (Fig. 1) (10, 13, 17, 33, 41). Several mechanisms could account for the new PLP biosynthesis pathway that bypasses the requirement for the PdxH oxidase in *pdxJ*(G194S) mutants. In this new pathway, the product of the ring closure must be PLP rather than PNP (Fig. 1). Our favored model

TABLE 1. *E. coli* K-12 strains and plasmids

Strain or plasmid	Genotype or phenotype[a]	Source or reference
Strains		
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA19* (Δ*lac-proAB*) (F′ *traD36 proAB lacI*^{q} *Z*ΔM15)	Promega Corp.
JC7623	*recB21 recC22 sbc-15 ara arg his leu pro thr*	A. J. Clark collection (15)
NU402	W3110 *pdxB*::Km^r (*Hin*dIII)	Laboratory collection (1)
NU812	W3110 *pdxA*::Km^r (*Eco*RV)	Laboratory collection (23)
NU816	W3110 *lacU169 tna2 sup*^{0}	C. Yanofsky collection
NU927	CGSC4556 *pdxJ19 srl*::Tn*10 recA1*	Laboratory collection (16)
NU1428	NU816 *recO*::Mu*dII* (Km^r)	Laboratory collection (16)
TX2767	JC7623 Δ*pdxH*::Ω(Cm^r)	Transformation of JC7623 by linearized pTX363
TX2768	NU816 Δ*pdxH*::Ω(Cm^r)	NU816 × P1*vir*(TX2767)
TX2773	TX2768 *pdxJ*(G194S)	2AP mutagenesis of TX2768
TX3547	TX2773 *pdxA*::Km^r	TX2773 × P1*vir*(NU812)
TX3548	TX2773 *pdxB*::Km^r	TX2773 × P1*vir*(NU402)
Plasmids		
pBR322	Medium-copy-number ColE1 replicon; Ap^r Tc^r	Laboratory collection (4)
pBR325	Medium-copy-number ColE1 replicon; Ap^r Tc^r Cm^r	Laboratory collection (3)
pGEM-3Z	High-copy-number ColE1 replicon; Ap^r	Promega Corp.
pUC18 and pUC19	High-copy-number ColE1 replicon; Ap^r	New England Biolabs, Inc.
pHP45-ΩCm	Plasmid containing Ω(Cm^r) cassette; Ap^r Cm^r	Prentki and Krisch (22)
pNU199	pBR322 containing 3.2-kb *Eco*RI-*Cla*I *pdxJ*$^+$ *dpj*$^+$ fragment	Laboratory collection (16)
pTX293	pBR325 containing 5.4-kb *Bam*HI-*Bam*HI *pdxH*$^+$ fragment; Ap^r Cm^r	Laboratory collection (18)
pTX335	pUC19 containing 1.5-kb *Hin*cII-*Hin*cII ORF1′-*pdxH*$^+$-*tyrS*′ fragment of pTX293; P_{lac}-*pdxH*$^+$; Ap^r	Laboratory collection (40)
pTX363	pTX335 Δ*pdxH*::Ω(Cm^r); *Dsa*I-*Dsa*I fragments in *pdxH* replaced with Ω(Cm^r) cassette; Ap^r Cm^r	This work
pTX366	pBR322 containing 10-kb fragment of TX2773 genomic DNA that suppressed the Δ*pdxH*::Ω(Cm^r) mutation; Ap^r	Transformation of TX2768 with TX2773 genomic library
pTX376[b]	pBR322 containing 979-bp *Nru*I-*Ssp*I P_{pdxJ}-*pdxJ*$^+$ fragment of pNU199; Ap^r	This work
pTX379[b]	pBR322 containing 979-bp *Nru*I-*Ssp*I P_{pdxJ}-*pdxJ*(G194S) fragment of pTX366; Ap^r	This work
pTX380[b]	pUC18 containing 979-bp *Nru*I-*Ssp*I P_{pdxJ}-*pdxJ*(G194S) fragment of pTX366; Ap^r	This work

[a] Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Tc^r, tetracycline resistant; Km^r, kanamycin resistance cassette (1, 2). *pdxJ*(G194S), G-to-A transition mutation that changes amino acid 194 in the PdxJ polypeptide from glycine to serine (see text).

[b] Minimal clones containing only the P_{pdxJ} promoter and the *pdxJ*$^+$ or *pdxJ*(G194S) coding sequence.

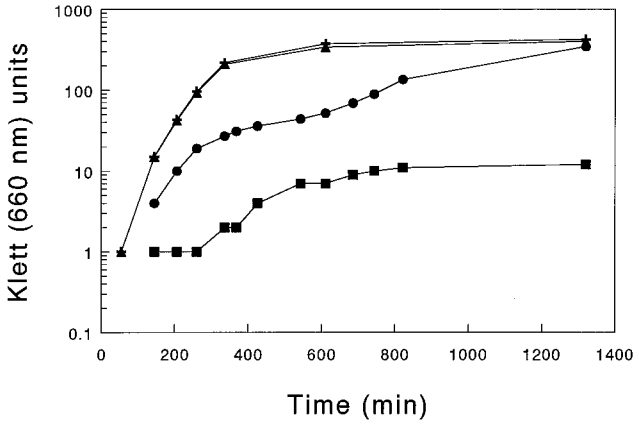

FIG. 3. Impaired growth of *pdxJ*(G194S) Δ*pdxH*::Ω(Cm^r) mutant TX2773 when PdxJ$^+$ is expressed from a multicopy plasmid. Bacterial strains were grown in liquid cultures of MM-glucose medium plus 0.5% (wt/vol) Casamino Acids and 25 μg of ampicillin per ml with shaking at 37°C. Symbols: +, NU816 (*pdxH*$^+$ *pdxJ*$^+$) carrying pBR322; ▲, NU816 carrying pTX376 (P_{pdxJ}-*pdxJ*$^+$ minimal clone); ●, TX2773 [Δ*pdxH*::Ω(Cm^r) *pdxJ*(G194S)] carrying pBR322; ■, TX2773 carrying pTX376.

assumes that wild-type PdxJ$^+$ normally brings DX into the ring closure reaction. DX contains the alcohol group that becomes the C-4′ alcohol group of PNP (Fig. 1). The ring closure scheme proposed by Hill and Spenser (12) and Kennedy et al. (13) does not involve any reactions at the carbon marked C-4′ in DX (Fig. 1). DX is likely formed by a transketolase reaction between D-glyceraldehyde and pyruvate (Fig. 1) (12, 36, 37). The PdxJ(G194S) mutant protein may bring the related compound 3-acetyl-glyceraldehyde (HCO-HCOH-HCOH-CO-CH_3; synonyms 2,3,-dihydroxylevulinic aldehyde or levulinaldehyde) instead of DX (H_2COH-HCOH-HCOH-CO-CH_3) into the ring closure reaction. 3-Acetyl-glyceraldehyde could be formed by a transketolase reaction between malonaldehyde (HCO-HCOH-HCO) and pyruvate, analogous to the one between D-glyceraldehyde and pyruvate (Fig. 1).

Alternatively, if DXP is used as a substrate, the wild-type PdxJ$^+$ protein may remove the phosphoric ester group during ring closure to form the C-4′ alcohol group. The PdxJ(G194S) mutant protein may remove the phosphoric ester group to leave a C-4′ aldehyde group instead of the alcohol. A less favored model assumes that the *pdxJ*(G194S) mutation turns the PdxJ protein into an activator of a cryptic pathway of PLP biosynthesis. This model is not supported by the fact that extensive mutagenesis, which was carried out to saturation for the *pdxJ*(G194S) mutation (above), did not turn up point mutations in genes that participate in such a hypothetical cryptic pathway. Testing these models awaits the development of an in vitro ring closure reaction system containing purified wild-type PdxJ$^+$ and mutant PdxJ(G194S) proteins.

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