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Identification and Function of the *pdxY* Gene, Which Encodes a Novel Pyridoxal Kinase Involved in the Salvage Pathway of Pyridoxal 5′-Phosphate Biosynthesis in *Escherichia coli* K-12

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pdxK **encodes a pyridoxine (PN)/pyridoxal (PL)/pyridoxamine (PM) kinase thought to function in the salvage pathway of pyridoxal 5′-phosphate (PLP) coenzyme biosynthesis. The observation that *pdxK* null mutants still contain PL kinase activity led to the hypothesis that *Escherichia coli* K-12 contains at least one other B_6-vitamer kinase. Here we support this hypothesis by identifying the *pdxY* gene (formally, open reading frame *f287b*) at 36.92 min, which encodes a novel PL kinase. PdxY was first identified by its homology to PdxK in searches of the complete *E. coli* genome. Minimal clones of *pdxY*$^+$ overexpressed PL kinase specific activity about 10-fold. We inserted an omega cassette into *pdxY* and crossed the resulting *pdxY*::ΩKan^r mutation into the bacterial chromosome of a *pdxB* mutant, in which de novo PLP biosynthesis is blocked. We then determined the growth characteristics and PL and PN kinase specific activities in extracts of *pdxK* and *pdxY* single and double mutants. Significantly, the requirement of the *pdxB pdxK pdxY* triple mutant for PLP was not satisfied by PL and PN, and the triple mutant had negligible PL and PN kinase specific activities. Our combined results suggest that the PL kinase PdxY and the PN/PL/PM kinase PdxK are the only physiologically important B_6 vitamer kinases in *E. coli* and that their function is confined to the PLP salvage pathway. Last, we show that *pdxY* is located downstream from *pdxH* (encoding PNP/PMP oxidase) and essential *tyrS* (encoding aminoacyl-tRNA^{Tyr} synthetase) in a multifunctional operon. *pdxY* is completely cotranscribed with *tyrS*, but about 92% of *tyrS* transcripts terminate at a putative Rho-factor-dependent attenuator located in the *tyrS-pdxY* intercistronic region.**

Pyridoxal 5′-phosphate (PLP) is the active form of vitamin B_6 and acts as an essential, ubiquitous coenzyme in many aspects of amino acid and cellular metabolism (3, 7, 10). PLP is synthesized de novo in *Escherichia coli* by a pathway that is thought to condense 4-phosphohydroxy-L-threonine (4PHT) and D-1-deoxyxylulose to form pyridoxine 5′-phosphate (PNP) (9, 12, 18–21, 25, 41, 45–47). PNP is then oxidized by the PdxH oxidase to form PLP, the active coenzyme (Fig. 1) (4, 24, 26, 27, 38, 48). In addition, PLP can be synthesized by a salvage pathway that utilizes pyridoxal (PL), pyridoxine (PN), and pyridoxamine (PM) taken up from the growth medium (Fig. 1) (20, 44). In the salvage pathway, PL, PN, and PM are first phosphorylated by kinases to form PLP, PNP, and pyridoxamine 5′-phosphate (PMP), respectively (Fig. 1). PNP and PMP are oxidized by the PdxH oxidase, which functions in both the salvage and de novo pathways (20, 26, 29, 48). Similar salvage pathways are present in mammalian cells, which lack a de novo PLP biosynthetic pathway (5, 6, 17). In mammalian cells, PLP homeostasis is further maintained by the offsetting activities of PL kinases and a PLP-specific phosphatase (13–15). A cytoplasmic PLP phosphatase activity has been detected in *E. coli* K-12, but it has not yet been determined whether this phosphatase is specific for PLP (43).

We recently reported the identification of the *pdxK* gene, which encodes a PN kinase (44). Previously, a PN kinase with additional PL and PM kinase activities was purified from *E. coli*, and it is likely that *pdxK* encodes this PN/PL/PM kinase (39). This was the first identification of a gene encoding a PN/PL/PM kinase in any organism and led to the rapid identification of a gene encoding a PL kinase in humans (17). A reverse genetics approach was used in the protozoan *Trypanosoma brucei* to identify a gene encoding a PL kinase, which showed significant homology to *E. coli* PdxK (34).

We showed previously that a *pdxK* null mutant lacks PN kinase activity but still contains PL kinase activity that is detectable in bacteria in which de novo PLP biosynthesis is blocked (44). This finding led to the hypothesis that *E. coli* K-12 contains at least one other PL kinase that converts PL to PLP. Here we confirm this hypothesis by identifying the *pdxY* gene, which encodes a novel PL kinase whose function is confined to the B_6 vitamer salvage pathway. We show further that *pdxY* is located in a multifunctional operon that contains the gene for the PdxH PNP/PMP oxidase, which functions in both the de novo and salvage pathways of PLP synthesis (Fig. 1), and the essential *tyrS* gene, which encodes aminoacyl-tRNA^{Tyr} synthetase.

MATERIALS AND METHODS

Materials. Restriction endonucleases, T4 DNA polymerase, T4 DNA ligase, T7 RNA polymerase, SP6 RNA polymerase, RQ1 DNase, and Wizard Miniprep DNA Purification Systems were purchased from Promega Corp. (Madison, Wis.). Some restriction endonucleases, Vent DNA (*exo*$^+$) polymerase, and 10× PCR buffer were purchased from New England Biolabs, Inc. (Beverly, Mass.). PN, PL, PM (98% pure), PLP, glycolaldehyde (GA), antibiotics, hydroxylamine, and zinc chloride were purchased from Sigma Chemical Co. (St. Louis, Mo.). RNase T2 and custom DNA oligomers were purchased from Gibco-BRL, Inc. (Gaithersburg, Md.). 4-Hydroxy-L-threonine (4HT) was a generous gift from Ian Spenser (McMaster University, Hamiton, Ontario, Canada). Bacto-Agar was obtained from Difco Laboratories (Detroit, Mich.). [^{3}H]PN substrate was synthesized by reduction of PL with sodium [^{3}H]borohydride as described previously

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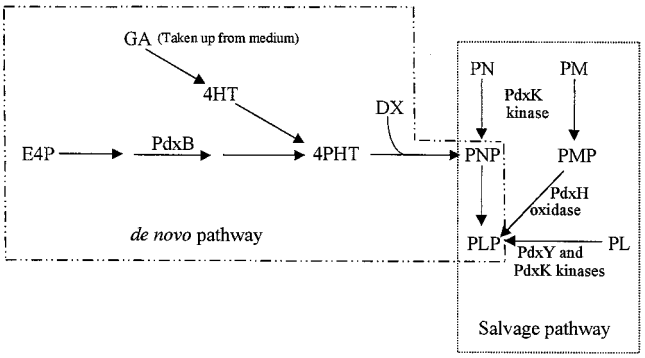

FIG. 1. De novo and salvage pathways for PLP biosynthesis in *E. coli* K-12. The de novo pathway illustrates that the intermediate 4PHT is synthesized from erythrose 4-phosphate (E4P) by a series of steps, one of which is catalyzed by the PdxB dehydrogenase (9, 25, 32). 4PHT can be produced in *pdxB* mutants from GA or 4HT by alternative pathways that normally do not contribute to de novo PLP biosynthesis (12, 20, 46). PNP, which is the first B_6 vitamer synthesized by the de novo biosynthetic pathway, is formed by the condensation of 4PHT and D-1-deoxyxylulose (DX) (18, 20, 21, 46). PNP formation from the de novo pathway does not require the activities of PL and PN kinases, which phosphorylate PL, PN, and PM taken up from the environment. The PNP/PMP oxidase PdxH functions in both the de novo and salvage pathways. As shown here, PdxY is a PL kinase in vivo, whereas PdxK is a PN kinase that can also phosphorylate PL and PM. See the text for additional details.

(44). [α-^{32}P]CTP (10 mCi/ml; >4,000 Ci/mmol) used to radiolabel RNA probes was purchased from Amersham Corp. (Arlington Heights, Ill.).

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are listed in Table 1. Strains isogenic to NU426 were constructed by generalized transduction with P1*vir* bacteriophage (28, 35). Cloning and genetic manipulations were performed by standard methods (31).

Vogel-Bonner minimal salts (1 × E) plus 0.4% (wt/vol) glucose medium (MMG) was supplemented with 1 μM PN, 1 μM PL, 1 μM PM, 1 mM GA, or 6 μM 4HT where indicated. Solid medium contained 1.5% (wt/vol) Bacto Agar. Luria-Bertani (LB) medium (10 g of NaCl per liter) was prepared from capsules purchased from Bio 101, Inc. (Vista, Calif.). Antibiotics were added to MMG and LB at the following concentrations where indicated: kanamycin, 12.5 and 50 μg per ml, respectively; chloramphenicol, 20 and 25 μg per ml, respectively; tetracycline, 10 μg/ml; and ampicillin, 50 to 100 μg per ml.

Cloning of *pdxY*. Putative genes encoding pyridoxal kinases were identified by Blast searches of the complete *E. coli* genome for homologs of *pdxK* (see Results and Discussion). A hypothetical reading frame (*f287b*) of 287 amino acids was found to encode PL kinase and was designated *pdxY*. It was amplified on 1.1- and 1.6-kb fragments from *E. coli* genomic DNA by using standard PCR with Vent (*exo*$^+$) DNA polymerase. The primers used to amplify *pdxY* on the 1.1- and 1.6-kb fragments were S1 (5′-AGAAGCTTGTCTGTTTGGTCGTTTTA-3′ in *tyrS*)/S2 (5′-AACTGAATTCGGAAGGGTTAGAGCAC-3′ in *gst*) and L1 (5′-TGCAAGCTTCCCGTGGTCAGGCA-3′ in *tyrS*)/L2 (5′-CCTGAATTCCTGCTGGATGACGGTA-3′ in *gst*), respectively. The S1 and L1 or S2 and L2 primers contain *Hin*dIII or *Eco*RI sites, respectively. The PCR fragments were ligated into the *Hin*dIII and *Eco*RI sites of vector pUC19 to form plasmids pTX608 and pTX618 (Table 1; Fig. 2). The orientation of the *pdxY* reading frame was the same as that of the *lacZ*α segment in the pUC19 vector, and isopropyl-β-D-thiogalactopyranoside (IPTG) induced *pdxY* expression (see Results and Discussion).

Culture growth, preparation of S150 crude extracts, and enzyme assays. Five milliliters of starter cultures containing LB medium, the supplements indicated,

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

Strain or plasmid[a]	Genotype or description[b]	Source or reference
Strains		
DH5α	F$^-$ ϕ80*dlacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *supE44 relA1 deoR*Δ(*lacZYAargF*) *U169*	Lab stock
JC7623	*recB21 recC22 sbc-15 ara arg his leu pro thr*	A. J. Clark (23)
JM109	*endA1 recA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *relA1 supE44* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}*Z*ΔM15]	Promega, Inc.
NU426	W3110 prototroph [W3110 *sup*(Am); probably W1485E]	C. Yanofsky (2)
NU402	NU426 *pdxB*::ΩKan^r	Lab stock (1)
NU816	W3110 *tnaA2* Δ*lacU169*	C. Yanofsky (2)
NU1018	NU814 *pdxB*::mini-Tn*10*Tet^r	Lab stock used in cross
TX2768	NU816 *pdxH*::ΩCm^r	Lab stock (26)
TX3632	NU426 *pdxB*::ΩKan^r *pdxK*::mini-Tn*10*Cm^r	Lab stock (44)
TX3634	NU426 *pdxB*::ΩKan^r *pdxK*::mini-Tn*10*Cm^r	NU402 × P1*vir*(TX3632)
TX3689	NU426 *pdxK*::mini-Tn*10*Cm^r	NU426 × P1*vir*(TX3632)
TX4015	NU426 *pdxB*::mini-Tn*10*Tet^r	NU426 × P1*vir*(NU1018)
TX4016	NU426 *pdxB*::mini-Tn*10*Tet^r *pdxK*::mini-Tn*10*Cm^r	TX3634 × P1*vir*(NU1018)
TX4017	JC7623 *pdxY*::ΩKan^r	JC7623 × linear pTX623
TX4021	NU426 *pdxY*::ΩKan^r	NU426 × P1*vir*(TX4017)
TX4022	NU426 *pdxB*::mini-Tn*10*Tet^r *pdxY*::ΩKan^r	TX4015 × P1*vir*(TX4017)
TX4023	NU426 *pdxK*::mini-Tn*10*Cm^r *pdxY*::ΩKan^r	TX3689 × P1*vir*(TX4017)
TX4024	NU426 *pdxB*::mini-Tn*10*Tet^r *pdxK*::mini-Tn*10*Cm^r *pdxY*::ΩKan^r	TX4016 × P1*vir*(TX4017)
Plasmids		
pHP45-ΩKan^r	Plasmid carrying ΩKan^r cassette	P. Prentki and H. M. Krisch (30)
pTX303	0.57-kb *Ava*I-*Sac*II fragment from pTX281 cloned into the *Bam*HI site of pGEM3Z; source of RNA probes 1 and 1o	Lab stock (24)
pTX485	*pdxK*$^+$ clone in pGEM3Z vector; *pdxK* transcription direction opposite to that of *lacZ*	Lab stock (44)
pTX608	1.1-kb minimal *pdxY*$^+$ clone in pUC19 vector; *pdxY* transcription in same orientation as *lacZ*	This work
pTX618	1.6-kb *pdxY*$^+$ clone in pUC19 vector; *pdxY* transcription in same orientation as *lacZ*	This work
pTX623	pTX618 *pdxY*::ΩKan^r	This work
pTX628	1.5-kb *Ava*I-*Eco*RV fragment from pNU217 (24) cloned into *Ava*I and *Hin*cII sites of pGEM3Z; source of RNA probes 2 and 2o	This work
pTX629	0.60-kb *Eco*RI-*Pst*I fragment from pTX630 cloned into *Eco*RI and *Pst*I sites of pGEM3Z; source of RNA probes 5 and 5o	This work
pTX630	1.6-kb *pdxY*$^+$ clone in pGEM3Z vector; source of RNA probes 4 and 4o	This work
pTX632	Self-ligation of 2.7-kb fragment from the *Ava*I and *Bam*HI double digestion of pTX628; source of RNA probes 3 and 3o	This work

[a] See Materials and Methods for additional details about strain and plasmid constructions.

[b] Kan^r, Tet^r, and Cm^r, resistance to kanamycin, tetracycline, and chloramphenicol, respectively. Besides the antibiotic resistances indicated, all plasmids also imparted resistance to ampicillin (Ap^r).

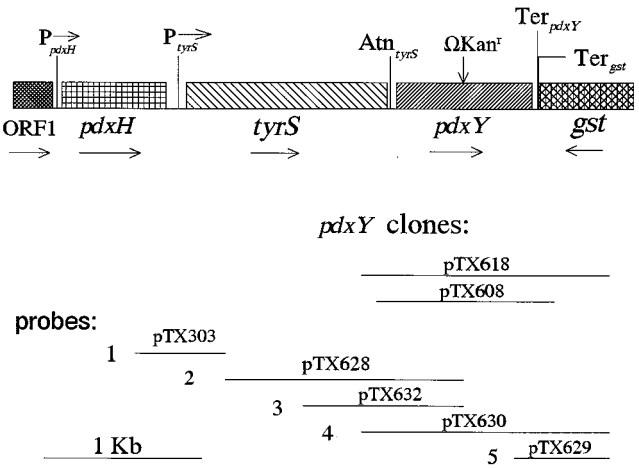

FIG. 2. Structure and transcription of the region surrounding the *pdxY* gene at 36.9 min in the chromosome of *E. coli* K-12. The figure is drawn to scale. The orientations of the reading frames of *pdxH* (encoding PNP/PMP oxidase), *tyrS* (encoding tyrosine aminoacyl-tRNA synthetase), and *gst* (encoding glutathione *S*-transferase) and the location of the *pdxY*::ΩKan^r insertion constructed herein are indicated by arrows. Locations of the P_{pdxH} and P_{tyrS} promoters are from reference 24, and the Rho-factor-dependent Atn_{tyrS} attenuator and Ter_{pdxY} and Ter_{gst} terminators were localized as described in the text. Horizontal lines represent the *pdxY*$^+$ inserts, generated by high-fidelity PCR, used to construct plasmids pTX618 and pTX608 (minimal *pdxY*$^+$ clone) and the inserts in the indicated plasmids used to synthesize RNA probes 1 to 5 and 1o to 5o for mapping of in vivo transcripts by RNase T2 protection assays (see the text and Materials and Methods).

and appropriate antibiotics were grown overnight at 37°C with vigorous shaking. One to two milliliters of the overnight cultures was inoculated into 200 ml of fresh LB containing the indicated supplements. Antibiotics were omitted from final cultures except for maintaining plasmids, in which case 50 to 100 μg of ampicillin per ml was added. The cultures were grown with shaking at 37°C to a turbidity of 70 Klett (660 nm) units (≈6.1×10^8 cells per ml) and were harvested by centrifugation at 4,420 × *g* for 15 min at 4°C. Cells were resuspended in 20 ml of cold 20 mM KPO_4 buffer (pH 7.2) and were collected by centrifugation. Following a second round of washing, pellets were resuspended in 2 ml of cold 40 mM KPO_4 buffer (pH 7.2) containing 1 mM dithiothreitol. Cells were disrupted by passage through a French pressure cell (20,000 lb/in^2), and suspensions were centrifuged at 150,000 × *g* for 60 min. The S150 supernatants were assayed for PL and PN kinase activities.

PL kinase activity in S150 crude extracts was measured by a fluorometric assay as described in reference 36. Briefly, reaction mixtures (3 ml) contained 40 mM KPO_4 (pH 7.2), 0.1 mM PL, 1 mM ATP, 1 mM dithiothreitol, 0.5 mM zinc chloride, and about 2 mg of S150 crude extract. Reactions were started by addition of the S150 crude extract, and reaction mixtures were incubated at 37°C for 60 min. Hydroxylamine was then added to a final concentration of 1 mM, and fluorescence intensity (excitation wavelength, 380 nm; emission wavelength, 450 nm) was determined 90 s later to maximize the signal-to-background ratio of hydroxylamine adducts of PLP over PL. The combined fluorescence intensities of control reaction mixtures lacking either crude extract or PL were subtracted for each sample. Protein concentrations were determined by using a Bradford Protein Assay Kit with bovine serum albumin as the standard (Bio-Rad, Inc., Torrance, Calif.). The linearity of the PL kinase assay was confirmed for incubations of at least 60 min and for reaction mixtures containing 1 to 4.5 mg of S150 crude extract (data not shown).

PN kinase activity in S150 crude extracts was determined by conversion of [^{3}H]PN to [^{3}H]PNP as described before (44), except that the specific activity of the [^{3}H]PN substrate was decreased to 3.8 mCi/mmol.

Construction of a *pdxY*::ΩKan^r mutant. pTX618 was digested with *Eco*RV, which cuts in the middle of the *pdxY* reading frame (Fig. 2). An ΩKan^r cassette was isolated from a *Bam*HI and *Sca*I digestion of plasmid pHP45ΩKan^r (30), and the *Bam*HI ends of the cassette were made blunt by filling them in with T4 DNA polymerase. A ligation mixture containing the digested pTX618 and ΩKan^r cassette was used to transform strain DH5α, and transformants were selected on LB medium containing ampicillin and kanamycin. Restriction analysis of plasmid pTX623 purified from one transformant confirmed the location of the ΩKan^r cassette in the middle of the *pdxY* gene.

The *pdxY*::ΩKan^r insertion mutation was crossed into the bacterial chromosome by transforming strain JC7623 with pTX623 that was linearized by digestion with *Eco*RI (2, 40). One transformant, designated TX4017 (Table 1), that grew on LB medium containing kanamycin contained no plasmids and was sensitive to ampicillin. The location of the *pdxY*::ΩKan^r insertion at the expected location in the chromosome (36.92 min) (Fig. 2) was confirmed by TX2768 (*pdxH*::ΩCm^r) × P1*vir*(TX4017) crosses which showed that the Kan^r marker was 100% linked to the Cm^r marker in *pdxH*. The *pdxY*::ΩKan^r cassette insertion mutation was moved from TX4017 into strains NU426, TX3689, TX4015, and TX4016 by P1*vir* transduction to form strains TX4021, TX4023, TX4022, and TX4024, respectively (Table 1).

RNase T2 protection assay. Total RNA was purified from 15-ml cultures grown in LB medium at 37°C to a turbidity of 50 Klett (660 nm) units. RNase T2 protection assays were performed as described before (37). RNA probes 1 to 5 and complementary probes 1o to 5o (Fig. 2) were synthesized in vitro by using the following phage RNA polymerases (RNAP) and linearized plasmid templates: for probe 1, T7 RNAP and pTX303 cut with *Hin*dIII; for probe 1o, SP6 RNAP and pTX303 cut with *Eco*RI; for probe 2, SP6 RNAP and pTX628 cut with *Eco*RI; for probe 2o, T7 RNAP and pTX628 cut with *Hin*dIII; for probe 3, SP6 RNAP and pTX632 cut with *Eco*RI; for probe 3o, T7 RNAP and pTX632 cut with *Hin*dIII; for probe 4, T7 RNAP and pTX630 cut with *Hin*dIII; for probe 4o, SP6 RNAP and pTX630 cut with *Eco*RI; for probe 5, T7 RNAP and pTX629 cut with *Hin*dIII; and for probe 5o, SP6 RNAP and pTX629 cut with *Eco*RI. Protected regions of probes were analyzed by electrophoresis on gels containing 7 M urea and 6% polyacrylamide (37). No self-protection of any probes was detected for control hybridizations that contained tRNA instead of mRNA. Radioactivity in bands on dried gels was measured directly by using an Instant Imager (Packard Instrument Co., Meriden, Conn.). Sizes of protected fragments were estimated from standard curves of mobility versus size for RNA standards of known lengths, as described before (37).

RESULTS AND DISCUSSION

Identification of *pdxY* encoding PL kinase. We ran Blast searches of the recently reported *E. coli* genome to identify homologs of *pdxK* that might encode a missing PL kinase. These searches turned up three candidates: *f287b* (at 36.92 min; encoding a product 30% identical and 42% similar to PdxK over its whole length); *yeiI* (at 48.49 min; encoding a product 19% identical and 29% similar to PdxK over its whole length), and *yeiC* (at 48.63 min; encoding a product 20% identical and 35% similar to PdxK over its whole length). We amplified each of these reading frames by PCR under conditions that minimize errors and cloned them downstream of the P_{lac} promoter in the high-copy-number vector pUC19. We induced expression of these reading frames by addition of IPTG to cultures of cells containing the clones, and we determined PL kinase specific activity in cell crude extracts of several different clones of each construct (Table 2) (Materials and Methods). Minimal clones, such as pTX608 (Table 1; Fig. 2), containing the *f287b* reading frame in a 1.1-kb fragment and clones containing *f287b* in a slightly larger, 1.6-kb fragment, such as pTX618 (Table 1; Fig. 2), increased PL kinase specific activity 8- to 11-fold (Table 2, JM109 strains). This result suggested that *f287b* encoded a PL kinase, and we renamed the reading frame *pdxY*. Clones containing the *yeiI* and *yeiC* reading frames did not have increased PL kinase specific activity (data not shown) and were not studied further.

***pdxY* functions as a PL kinase in vivo.** We inserted an omega cassette into the *pdxY* reading frame and crossed the *pdxY*::ΩKan^r insertion mutation into the *E. coli* K-12 chromosome (Materials and Methods). We then moved the *pdxY*::ΩKan^r mutation into the isogenic strains listed in Tables 2 and 3. *pdxY*, *pdxK*, and *pdxY pdxK* mutants were not auxotrophs (TX3689, TX4021, and TX4023 [Table 3]). This result shows that the de novo pathway of PLP biosynthesis functions in the absence of the PN/PL/PM kinase PdxK and the PL kinase PdxY and is consistent with the model in which these kinases function solely in the salvage pathway (Fig. 1) (see below). In this model, the phosphate ester group of PNP, which is the first B_6 vitamer synthesized de novo, is provided by the intermediate 4PHT (46).

We next tested the growth requirements of *pdxY* and *pdxK* mutants in strains containing a *pdxB* mutation, which blocks the de novo PLP biosynthetic pathway upstream of 4PHT (Fig.

TABLE 2. PL and PN kinase specific activities in strains overexpressing the PdxY and PdxK proteins and in *pdxK*, *pdxY*, *pdxY pdxK*, and *pdxH* mutants[a]

Strain	Genotype or description	PL kinase sp act (pmol of PLP formed/min/mg of protein)[b]	PN kinase sp act (pmol of PNP formed/min/mg of protein)[b]
TX3977[c]	JM109(pUC19)	45 ± 9	ND
TX3981[c]	JM109(pTX608 *pdxY*$^+$)	371 ± 58	ND
TX4002[c]	JM109(pTX618 *pdxY*$^+$)	488 ± 21	ND
NU426[d]	*pdxB*$^+$ *pdxK*$^+$ *pdxY*$^+$ parent	72 ± 6	74 ± 16
NU402[e]	*pdxB pdxK*$^+$ *pdxY*$^+$	76 ± 15	75 ± 16
TX4015[f]	*pdxB pdxK*$^+$ *pdxY*$^+$	74 ± 6	86 ± 3
TX3634[e]	*pdxB pdxK pdxY*$^+$	80 ± 5	4 ± 1
TX4022[f]	*pdxB pdxK*$^+$ *pdxY*	26 ± 4	82 ± 6
TX4024[f]	*pdxB pdxK pdxY*	16 ± 1	1.5 ± 0.2
TX3636[e]	TX3634(pTX485 *pdxK*$^+$)	270 ± 41	320 ± 13
TX4037[c,f]	TX3634(pTX608 *pdxY*$^+$)	ND	57 ± 6
NU816[g]	*pdxB*$^+$ *pdxK*$^+$ *pdxY*$^+$ *pdxH*$^+$ parent	63 ± 8	ND
TX2768[g]	NU816 *pdxH*::ΩCm^r	47 ± 4	ND

[a] See Materials and Methods for growth conditions and description of PL and PN kinase assays.
[b] All values were determined by duplicate or triplicate assays of two or more independent cultures. Experimental errors are expressed as standard errors of the mean. ND, not determined.
[c] IPTG was added to a final concentration of 1 mM when the final cultures reached a density of 20 Klett (660 nm) units.
[d] GA (2 mM) or PL (2 μM) was added to overnight starter, and final cultures were assayed for both PL and PN kinase activities.
[e] PL (2 μM) was added to overnight starter, and final cultures were assayed for both PL and PN kinase activities.
[f] GA (2 mM) was added to overnight starter, and final cultures were assayed for both PL and PN kinase activities.
[g] PL (100 μM) was added to overnight starter, and final cultures were assayed for PL kinase activity.

1). *pdxB* mutants can synthesize 4PHT only when supplemented with GA or 4HT by an alternative pathway involving ThrB homoserine kinase (12, 46). The *pdxB pdxK pdxY*$^+$ mutant grew when supplemented with 1 μM PL, but not when supplemented with 1 μM PN, as shown previously (TX4016 [Table 3]) (44). Given that the B_6 vitamers are present in *E. coli* in relatively small amounts and need to be added as supplements (8, 9), this result confirms the conclusion that the PdxK gene product is the major PN kinase in *E. coli* K-12. Addition of 100 μM PN allowed growth of the *pdxB pdxK pdxY*$^+$ mutant; however, this result cannot be interpreted, because our high-performance liquid chromatographic analyses demonstrated that commercial PN contains a contaminant that could be PL at very low levels (data not shown). Likewise, growth tests with PM were inconclusive, because commercial PM is contaminated with as much as 2% (wt/wt) PL. The *pdxB pdxK*$^+$ *pdxY* mutant grew when supplemented with PN or PL (TX4022 [Table 3]), consistent with the previous conclusion that the purified PdxK enzyme possesses PL, as well as PN, kinase activity (39). Most significantly, the PLP requirement of the *pdxB pdxK pdxY* triple mutant was not satisfied by PN, PL, or PM, and the triple mutant grew only when supplemented with GA or 4HT (TX4024 [Table 3]). This result shows that PdxK and PdxY are the only physiologically significant PL, PN, and PM kinases in *E. coli*. Moreover, growth of the *pdxB pdxK pdxY* triple mutant on GA and 4HT, but not on PL, PN, or PM, strongly supports the model in which B_6 vitamer kinases participate only in the salvage pathway of PLP biosynthesis (Fig. 1).

PL and PN kinase assays of crude extracts (Table 2) gave results consistent with the conclusions from the growth experiments (Table 3). The *pdxB pdxK*$^+$ *pdxY*$^+$ mutant had the same PL and PN kinase specific activities as the *pdxB*$^+$ parent strain (NU426, NU402, and TX4015 [Table 2]). The *pdxB pdxK pdxY*$^+$ double mutant lacked significant PN kinase activity but contained unchanged PL kinase specific activity (NU426 and TX3634 [Table 2]). Compared to the *pdxB pdxK*$^+$ *pdxY*$^+$ parent, the PL kinase specific activity was reduced about threefold in the *pdxB pdxK*$^+$ *pdxY* double mutant, which still had full PN kinase activity (TX4015 and TX4022 [Table 2]). Finally, the *pdxB pdxK pdxY* triple mutant lacked PN kinase activity and contained reduced PL kinase activity compared to the *pdxB pdxK*$^+$ *pdxY* double mutant (TX4015, TX4022, and TX4024 [Table 2]). The apparent residual PL and PN kinase activities in the *pdxB pdxK pdxY* triple mutant were not physiologically significant, because the triple mutant failed to grow when supplemented with PN, PL, or PM (Table 3 and data not shown). These residual activities may simply reflect background in the enzyme assays containing crude extracts. In particular, the PL kinase assay has a high background, because hydroxylamine forms fluorescent oxime adducts at a slower rate with the PL substrate than with the PLP product (36). A residual background could result if the formation of PL-oxime was slightly greater in reaction mixtures containing crude extract than in control mixtures lacking extract. Alternatively, other carbohydrate kinases, which are evolutionarily related to PdxK and PdxY (39), may use PL and PN at low levels in in vitro enzyme assays. Consistent with this notion, bacteria containing suppressors of the *pdxB pdxK pdxY* triple mutant readily appear on MMG plates supplemented with PN, PL, or PM at 37°C (43).

We further tested the conclusion that PdxK functions as both a PL kinase and a PN kinase by overexpressing the PdxK protein. The PN and PL kinase specific activities in crude extracts increased 80- and 3-fold, respectively, in the strain overexpressing PdxK from plasmid pTX485 compared to the *pdxB pdxK pdxY*$^+$ strain lacking the plasmid (TX3634 and TX3636 [Table 2]). We also tested whether the PdxY kinase possessed a low-level PN kinase. Overexpression of PdxY did increase PN kinase about 10-fold over the background level in the *pdxB pdxK pdxY*$^+$ strain (TX3634 and TX4037 [Table 2]) and allowed growth on MMG containing 2 μM PN (data not shown). However, the PdxY PN kinase activity was not physiologically significant under the growth conditions tested, because a *pdxB pdxK pdxY*$^+$ mutant failed to grow when supplemented with

TABLE 3. Growth properties of *pdxB*, *pdxK*, and *pdxY* single, double, and triple mutants on supplemented MMG[a]

Strain	Supplement added to MMG				
	None	PN	PL	GA	4HT
NU426 (parent)	+	+	+	+	+
TX3689 (*pdxK*)	+	+	+	+	+
TX4021 (*pdxY*)	+	+	+	+	+
TX4023 (*pdxK pdxY*)	+	+	+	+	+
TX4015 (*pdxB*)	−	+	+	+	+
TX4016 (*pdxB pdxK*)	−	−	+	+	+
TX4022 (*pdxB pdxY*)	−	+	+	+	+
TX4024 (*pdxB pdxK pdxY*)	−	−	−	+	+

[a] Bacteria were streaked from LB plates containing 1 mM GA and appropriate antibiotics onto freshly prepared MMG plates containing the indicated supplements. Plates were incubated at 37°C for about 40 h before growth was scored. Supplements were added at the following final concentrations: PN, 1 μM; PL, 1 μM; GA, 1 mM; 4HT, 6 μM. Commercial PM is only about 98% pure and gave the same results as PL (data not shown). +, confluent growth in heavily streaked areas and formation of single colonies; −, no single-colony formation.

PN (Table 3). Thus, the combined amino acid homology, growth, and enzyme assay data support the conclusions that *pdxK* encodes a PN kinase with moderate PL kinase activity and *pdxY* encodes a PL kinase with a low level of PN kinase activity that can be detected when PdxY is overexpressed.

Comparisons of PdxY and PdxK with other B_6-vitamer kinases. Previously, we made the observation that the PdxK kinase was a member of a superfamily of carbohydrate kinases that includes phophofructokinases and ribokinases (44). This finding was unexpected because of the different structures of the carbohydrates and the substituted pyridine ring of the B_6 vitamers. Figure 3 presents an updated alignment that includes the *E. coli* PN/PL/PM kinase PdxK and the *E. coli* PL kinase PdxY, PL kinases from humans and *T. brucei* (17, 34), and proteins from *Haemophilus influenzae*, *Caenorhabditis elegans*, *Rattus norvegicus*, *Saccharomyces cerevisiae*, and *Salmonella typhimurium* that likely are PL or PN kinases. This alignment indicates conserved motifs that may be involved in substrate binding or catalysis, including signature motifs found in the PfkB superfamily of carbohydrate kinases (regions 1 and 4) (42, 44); degenerate P-loop motifs (regions 1 and 4), which may be involved in ATP binding (34); candidate tyrosine residues (marked 2), one of which cannot be modified following PL binding (33); candidate aspartic and glutamic acid residues (marked 3), which may act as general bases in phosphate transfer (34); and a region (marked 4) that is affinity labeled by the bisubstrate analog adenosine tetraphosphate in the PL kinase isolated from sheep brain (11). A degenerate Walker B motif located in the *T. brucei* PdxK kinase (near region 3 in the third panel of the alignment) was speculated to play a role in Mg^{2+} binding (34) but is not well conserved in the different B_6-vitamer kinases.

Dendrogams, phylograms, and cladograms of these sequences compiled by different evolution programs in the University of Wisconsin Genetics Computer Group package all suggest that the eukaryotic and the *H. influenzae* proteins are more closely related to PdxY than to PdxK, whereas the *S. typhimurium* protein, which is encoded from an analogous region of the chromosome, is more closely related to PdxK than to PdxY. Presumably, *S. typhimurium* contains an unidentified homolog of PdxY. The existence of PdxK and PdxY in *E. coli* raises the possibility that PN- and PL-specific kinases are present in other eubacteria and in eukaryotes. This possibility has implications for current efforts to exploit B_6 vitamer kinases in the uptake of selectively toxic analogs for the treatment of certain parasitic diseases (34). For example, it may be possible to exploit one of the two B_6 vitamer kinases present in some organisms to enhance the selectivity of drug uptake. Finally, it was recently communicated to us that the *E. coli* PN/PL/PM kinase PdxK also possesses a hydroxymethylpyrimidine kinase activity and therefore may function in thiamine (vitamin B_1) biosynthesis (22). It remains to be determined whether other PL and putative B_6 kinases (Fig. 3) have these dual functions that possibly allow cross talk between the vitamin B_6 and B_1 pathways.

Structure and expression of *pdxY*. *pdxY* is located at 36.9 min in the *E. coli* chromosome in the same orientation immediately downstream of *pdxH* (encoding PNP/PMP oxidase [Fig. 1]) and *tyrS* (encoding $tRNA^{Tyr}$-aminoacyl synthetase) and in the opposite orientation to *gst* (encoding glutathione *S*-transferase) (Fig. 2). Previously, we showed that *pdxH* and *tyrS* are transcribed from separate promoters in vivo, but about 20% of *tyrS* transcripts are present as *pdxH-tyrS* cotranscripts (24). No Rho-factor-independent terminators are obvious in the sequences of the *tyrS-pdxY* or *pdxY-gst* junctions (Fig. 2), and previously we did not detect transcription termination between *pdxH* and *tyrS* (24). Therefore, we mapped the *pdxY* transcript by RNase T2 protection assays (see Table 1 and Fig. 2 for probes) in order to determine the transcription relationship of these genes and to learn whether *pdxH* and *pdxY* are somehow cotranscribed.

Consistent with earlier results, hybridization to probe 1, corresponding to the *pdxH* and *tyrS* noncoding strand, showed two bands of 297 and 560 nucleotides (nt) (Fig. 4, lane 4), representing transcription from the P_{tyrS} and P_{pdxH} promoters, respectively (Fig. 2). Hybridization to probes 2 and 3, corresponding to the *tyrS* and *pdxY* noncoding strands, each gave a large, faint, protected band and a much more intense, smaller, protected band followed by degradation products (Fig. 4, lanes 7 and 10). The large, faint, protected bands are smaller than the undigested probes, which contain linker regions (Fig. 4, lanes 5 and 8), and represent contiguous *tyrS-pdxY* cotranscripts. Because probes 2 and 3 end at the same place in *pdxY* (Fig. 2), the intense, shorter 1,050- and 530-nt bands observed with probes 2 and 3, respectively, must correspond to terminated *tyrS* transcripts. The sizes of the bands place the termination point in the *tyrS-pdxY* intercistronic region about 20 nt downstream from the translation termination codon of *tyrS* (Fig. 2). No bands were detected in hybridizations with probes 1o, 2o, and 3o, corresponding to the *pdxH*, *tyrS*, and *pdxY* coding strand, indicating that there is no antisense transcription of *tyrS* and *pdxY* in vivo and no DNA contamination in our total-RNA preparations (data not shown).

Using probe 1, 2, or 3, we did not detect any protected band that would indicate the presence of an independent *pdxY* promoter. Therefore, *tyrS* and *pdxY* seemed to be cotranscribed, and the terminator between *tyrS* and *pdxY* (Atn_{tyrS}; Fig. 2) functioned as an attenuator to decrease *pdxY* expression relative to that of *tyrS*. To test further the transcription linkage between *tyrS* and *pdxY*, we tested whether a polar omega-cassette insertion mutation in *pdxH* decreased expression of the PdxY gene product. Previously we showed that blockage of transcription from P_{pdxH} by a *pdxH*::Mu*d*I-8 insertion reduced the *tyrS* transcript amount by about 20% (24). We found that a *pdxH*::ΩCm^r insertion also reduced PL kinase activity by about 25% (NU816 and TX2768 [Table 2]). This result is consistent with the interpretation that all *pdxY* transcription originates at the P_{pdxH} and P_{tyrS} promoters and that there is a low level of coupling between the transcription of *pdxH* and that of *pdxY*. Finally, quantitation of the radioactivity in gel bands indicated that about 92% of *tyrS* transcripts terminate at the Atn_{tyrS} terminator and only about 8% read through into *pdxY*. Since there is no Rho-factor-independent terminator structure in the *tyrS-pdxY* intercistronic region, Atn_{tyrS} is likely a Rho-factor-dependent transcription terminator.

The *tyrS-pdxY* cotranscript is terminated at a terminator (Ter_{pdxY}; Fig. 2) located in the *pdxY-gst* intercistronic region. Hybridization to probe 4, corresponding to the *pdxY* noncoding strand, gave 202- and 1,120-nt protected bands (Fig. 4, lane 21), which represent terminated *tyrS* transcripts at Atn_{tyrS} and *tyrS-pdxY* cotranscripts at Ter_{pdxY}, respectively. Hybridization to probe 5, corresponding to the *pdxY* noncoding strand, gave a faint 148-nt protected band (Fig. 4, lane 24) consistent with the location of Ter_{pdxY}, which is about 35 nt downstream from the *pdxY* translation stop codon (Fig. 2). Last, we located termination of the oppositely transcribed *gst* transcript at Ter_{gst} in the *pdxY-gst* intercistronic region about 4 nt downstream from the *gst* translation stop codon (Fig. 2). Hybridization to probes 4o and 5o, corresponding to the *gst* noncoding strand, gave 450-nt protected fragments representing termination at Ter_{gst} (Fig. 4, lanes 14 and 17). We also detected some (≈12%) readthrough of the Ter_{gst} terminator (Fig. 4, lane 14, series of

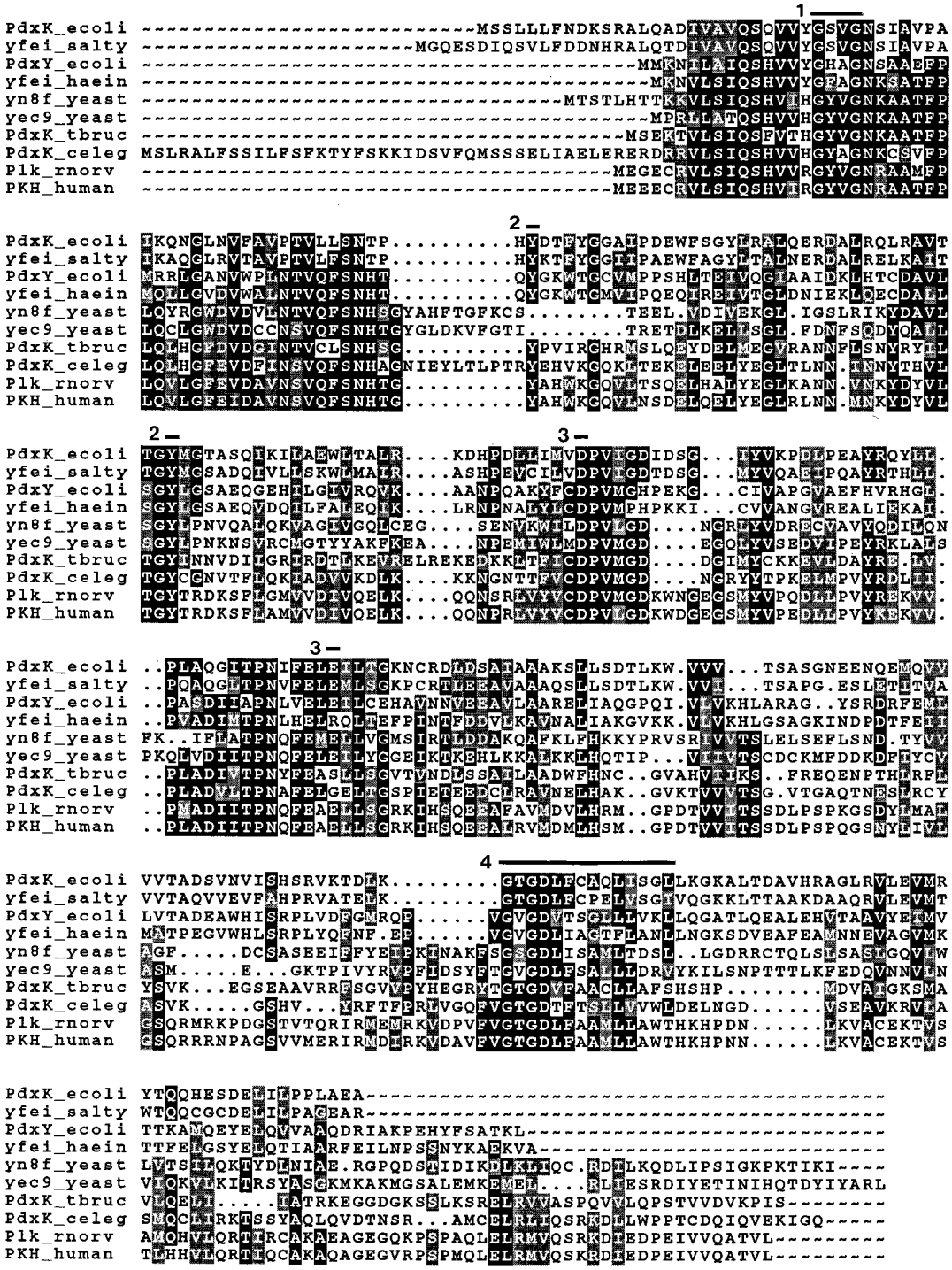

FIG. 3. Amino acid alignments of the *E. coli* PN/PL/PM kinase PdxK and the *E. coli* PL kinase PdxY with homologs from other organisms. Kinase activities have been demonstrated directly only for *E. coli* PdxK and PdxY (see the text) (44), human PKH (human homolog of pyridoxal kinase) (17), and *T. brucei* PdxK (34); the other sequences are putative homologs identified by Blast searches. The sequences have the following database accession numbers: *E. coli* PdxK (PdxK_ecoli), GenBank U53700; *S. typhimurium* Yfei (yfei_salty), SW P40192; *E. coli* PdxY (PdxY_ecoli), DDBJ D90807 cds10; *H. influenzae* Yfei (yfei_haein), SW P44690; *S. cerevisiae* Yn8fp (yn8f_yeast), SW P53727; *S. cerevisiae* Yec9p (yec9_yeast), SW P39988; *T. brucei* PdxK (PdxK_tbruc), GenBank U96712; *C. elegans* PdxK (PdxK_celeg), GenBank AF003142; *R. norvegicus* Plk (Plk_rnorv), GenBank AF020346; and human PKH (PKH_human), U89606. Conserved motifs that may be involved in substrate binding or catalysis are overlined and discussed in the text. Solid background, identical amino acids; shaded background, similar amino acids.

bands above the 450-nt protected fragment, and lane 17, upper band), which would produce an antisense *pdxY* transcript. However, as determined by the length of the read-through transcripts, this antisense transcription did not extend past the last quarter of the *pdxY* reading frame. This is consistent with our observation that no antisense *pdxY* transcript was detected with probes 1o, 2o, and 3o (see above). Thus, it is unlikely that an antisense *pdxY* transcript extends to the *pdxY* ribosome

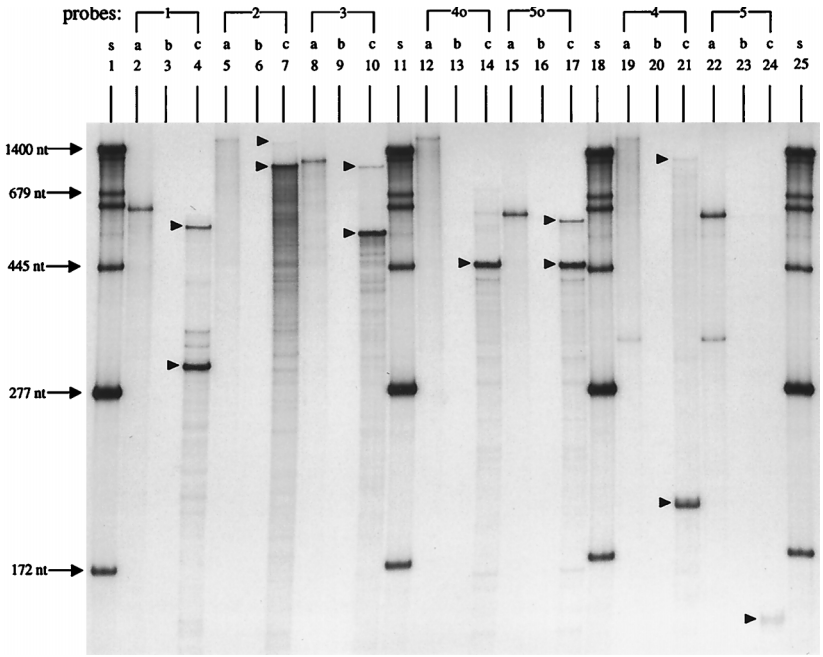

FIG. 4. RNase T2 protection assays of transcripts from the *pdxH-tyrS-pdxY-gst* region of the *E. coli* K-12 chromosome. Total RNA was purified from strain NU426 growing exponentially in LB medium at 37°C, and RNase T2 protection assays were performed as described in Materials and Methods. The positions of RNA probes 1 to 5 used for this assay are indicated in Fig. 2. Probes 1 to 5 correspond to the *pdxH*, *tyrS*, and *pdxY* noncoding strands and hybridized with *pdxH*, *tyrS*, and *pdxY* transcripts. Probes 4o and 5o are complementary to probes 4 and 5, respectively, and hybridized to *gst* transcripts. See the text for additional details. a, unhybridized RNA probes; b, RNA probes hybridized with 50 μg of tRNA (self-hybridization control); c, RNA probes hybridized with 50 μg of total cellular RNA; s, RNA size standards (see Materials and Methods) (37); arrowheads, protected RNA species described in Results and Discussion.

binding site. As is the case for Atn_{tyrS}, no Rho-factor-independent structures are obvious for Ter_{pdxY} and Ter_{gst}, and these terminators may be Rho factor dependent.

Detailed molecular genetic analyses have shown that genes encoding aminoacyl-tRNA synthetases are regulated by a variety of mechanisms in *E. coli*, including transcription (Ala-tRNA synthetase) and translation (Thr-tRNA synthetase) autoregulation and transcription attenuation (Phe-tRNA synthetase) (reviewed in reference 16). Currently, the regulation of *tyrS* in *E. coli* is largely unknown. Precedents from genes encoding other aminoacyl-tRNA synthetases in *E. coli* suggest that *tyrS* may be regulated positively by growth rate and by tyrosine limitation (16, 24). The cotranscription of *pdxY* and *tyrS* demonstrated here may provide a point of genetic integration that coordinates incorporation of amino acids into proteins with PLP coenzyme supply. Ongoing studies are aimed at elucidating the regulation and expression of the *pdxH-tyrS-pdxY* multifunctional operon and the roles of the PL kinase PdxY and the PL/PM/PN kinase PdxK in maintaining PLP homeostasis.

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