JOURNAL OF BACTERIOLOGY, Aug. 1998, p. 4294–4299 Vol. 180, No. 16
0021-9193/98/$04.00+0

Involvement of the *gapA*- and *epd* (*gapB*)-Encoded Dehydrogenases in Pyridoxal 5′-Phosphate Coenzyme Biosynthesis in *Escherichia coli* K-12

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We show that *epd* (*gapB*) mutants lacking an erythrose 4-phosphate (E4P) dehydrogenase are impaired for growth on some media and contain less pyridoxal 5′-phosphate (PLP) and pyridoxamine 5′-phosphate (PMP) than their *epd*$^+$ parent. In contrast to a previous report, we found that *gapA epd* double mutants lacking the glyceraldehyde 3-phosphate and E4P dehydrogenases are auxotrophic for pyridoxine. These results implicate the GapA and Epd dehydrogenases in de novo PLP and PMP coenzyme biosynthesis.

Pyridoxal 5′-phosphate (PLP) is an essential coenzyme used by many enzymes involved in amino acid metabolism and by glycogen phosphorylases (reviewed in references 7, 12, and 16). PLP is thought to be synthesized in *Escherichia coli* by the convergence of two pathways (Fig. 1) (10, 18, 19, 25). The two branches lead to the synthesis of 4-phosphohydroxy-L-threonine (4PHT) and 1-deoxy-D-xylulose 5-phosphate, which are condensed by the PdxA and PdxJ enzymes to form pyridoxine 5′-phosphate (PNP) (Fig. 1) (6, 23). PNP is oxidized by the PdxH enzyme to form the active PLP coenzyme (24, 29, 39) (Fig. 1). PLP is converted to pyridoxamine 5′-phosphate (PMP) by the half-reaction of transaminases (4, 12) (Fig. 1). PMP is recycled back to PLP by the second half-reaction of transaminases and by PdxH oxidase (29, 39).

Overwhelming genetic and biochemical evidence implicate 4PHT as an obligatory intermediate that provides the phosphate ester group of PNP (13, 35, 37). 4PHT biosynthesis is thought to start with D-erythrose-4-phosphate (E4P), which is oxidized by an E4P dehydrogenase to 4-phosphoerythronate (4PE) (Fig. 1, branch 1). 4PE is further oxidized by the PdxB dehydrogenase and transaminated by the SerC (PdxF) enzyme to form 4PHT (Fig. 1). Three pieces of evidence support this scheme. First, *tktA tktB* double mutants, which lack transketolase activity and cannot synthesize E4P or the six aromatic amino acids and vitamins (Fig. 1), are pyridoxine (PN) auxotrophs (38). Second, purified PdxB enzyme oxidizes 4PE in a nonsustained reaction (36). Last, the SerC (PdxF) enzyme uses 4PHT as a substrate in the reverse transamination reaction (13). As expected from this scheme, *pdxB* and *serC* (*pdxF*) mutants are PN auxotrophs, but no single mutation that blocked the first E4P dehydrogenase step in branch 1 of the pathway was identified (10, 23).

Previous studies of *epd* (*gapB*). We proposed and confirmed that the *gapB* gene, which we renamed *epd*, encoded a nonphosphorylating E4P dehydrogenase (36). The E4P dehydrogenase activity of the Epd enzyme was verified by Boschi-Muller et al. (5), who further identified amino acids in Epd required for E4P dehydrogenase activity and showed that Epd has low-level phosphorylating and nonphosphorylating glyceraldehyde 3-phosphate (G3P) dehydrogenase activities in the presence and absence of inorganic phosphate, respectively. However, this G3P dehydrogenase activity is not sufficient to allow the growth of *gapA* mutants, which lack the major G3P dehydrogenase of *E. coli* (9, 20). Conversely, Boschi-Muller et al. showed that the GapA dehydrogenase of *Bacillus stearothermophilus* has a low level of phosphorylating E4P dehydrogenase activity, but this result was not extended to the *E. coli* GapA enzyme (5).

Recently Della Seta et al. (9) reported that *E. coli gapB* single and *gapA gapB* double mutants do not show a growth requirement for B_6 vitamers, such as PN and pyridoxal (PL), which can be converted to PLP and PMP by a salvage pathway (34, 35). If this finding were correct, then it would argue against a requirement for an E4P dehydrogenase in PLP biosynthesis (Fig. 1). We performed experiments similar to those of Della Seta et al. using what should be equivalent strains in three different *E. coli* genetic backgrounds. In contrast to their results, we found that *gapA gapB* mutants are indeed auxotrophic for B_6 vitamers and that *gapB* mutants are impaired for growth without PN under some growth conditions.

Construction of *gapA* and *epd* mutants. We moved the prototypic *gapA1* point mutation of strain DF220 (20) by cotransduction with a linked Tn*10* transposon into prototrophic strains W3110 and MG1655 grown in Luria-Bertani (LB) medium containing 1% (vol/vol) glycerol plus 1% (wt/vol) succinate (Table 1). The resulting MG1655 *gapA1* mutant TX4125 had the expected phenotype of growth on plates containing minimal salts medium (MM) supplemented with glycerol plus succinate but no growth in 3 days on MM containing 0.4% (wt/vol) glucose (Table 2) (9, 20). The W3110 *gapA1* mutant TX3484 grew very slowly on MM containing glucose, suggesting slight leakiness of the *gapA1* mutation in some genetic backgrounds (Table 2); however, leakiness would not lead to the PN auxotrophy described below. Della Seta et al. mentioned low infectivity by P1 bacteriophage and spontaneous lysis of their Δ*gapA*::Cm mutants (9); we did not encounter similar difficulties with *gapA1* mutants.

We inserted an omega cassette imparting chloramphenicol resistance (Cm^r) into the single *Cla*I site of *epd* (*gapB*) and crossed the resulting *epd*::Ω(Cm^r) mutation, which also con-

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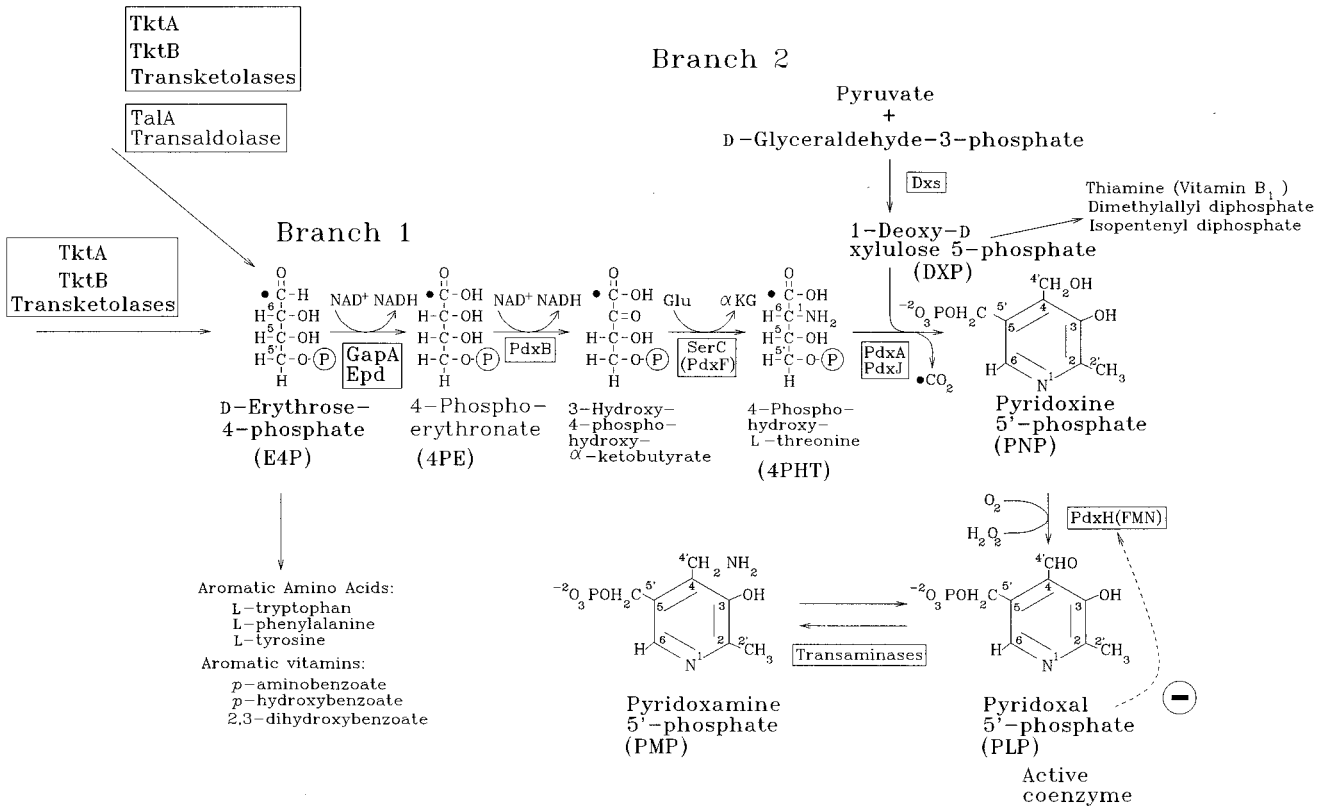

FIG. 1. Pathway for PLP and PMP coenzyme biosynthesis in *E. coli* K-12. Enzymes that catalyze the steps in the pathway are indicated by their genetic symbols and are boxed. Branch 1 takes E4P to 4PHT, and branch 2 provides DXP, which is condensed with 4PHT to form PNP. PNP is oxidized to the active coenzyme PLP, which can be converted to PMP by transaminases. Oxidation of E4P to 4PE is the first step of branch 1 and is catalyzed by the E4P dehydrogenase activities of the GapA and Epd (GapB) enzymes. See text for details.

TABLE 1. Strains and plasmids

Strain or plasmid	Genotype[a]	Source or reference
E. coli K-12 strains		
CAG12074	MG1655 *zea-3068*::Tn*10*	32
DF220[b]	*gap-1* (called *gapA1* here to avoid confusion with *gapB*)	20
DF221[b]	*gap-2* (called *gapA2* here to avoid confusion with *gapB*)	20
DH5α	*F′ endA1 hsdR17 supE44 thi-1 recA1 gyrA relA* Δ(*lacZYA-argE*)*U169 deoR*	New England Biolabs
JC7623	*recB21 recC22sbc15 ara arg his leu pro thr*	22
MG1655	Prototroph	3
NU816	W3110 *lacU169 tna2 sup*^{0} prototroph	C. Yanofsky
TX3446[c]	JC7623 *epd* (*gapB*)::Ω(Cm^r)	Transformation with pTX457 cut with *Sal*I and *Sph*I
TX3457[c]	NU816 *epd* (*gapB*)::Ω(Cm^r)	NU816 × P1*vir*(TX3446)
TX3470	NU816 *zea-3068*::Tn*10*	NU816 × P1*vir*(CAG12074)
TX3481[c]	NU816 *epd* (*gapB*)::Ω(Cm^r) *zea-3068*::Tn*10*	TX3457 × P1*vir*(CAG12074)
TX3459	DF220 *zea-3068*::Tn*10*	DF220 × P1*vir*(CAG12074)
TX3484	NU816 *gapA1 zea-3068*::Tn*10*	NU816 × P1*vir*(TX3459)
TX3491[c]	NU816 *epd* (*gapB*)::Ω(Cm^r) *gapA1 zea-3068*::Tn*10*	TX3457 × P1*vir*(TX3459)
TX4074[c]	MG1655 *epd* (*gapB*)::Ω(Cm^r)	MG1655 × P1*vir*(TX3481)
TX4090	MG1655 *zea-3068*::Tn*10*	MG1655 × P1*vir*(TX3481)
TX4092[c]	MG1655 *epd* (*gapB*)::Ω(Cm^r) *zea-3068*::Tn*10*	TX4074 × P1*vir*(TX3481)
TX4125	MG1655 *gapA1 zea-3068*::Tn*10*	MG1655 × P1*vir*(TX3484)
TX4134[c]	MG1655 *epd* (*gapB*)::Ω(Cm^r) *gapA1 zea-3068*::Tn*10*	TX4074 × P1*vir*(TX3484)
TX4187	DF221 *epd* (*gapB*)::Ω(Cm^r)	DF221 × P1*vir*(TX3481)
Plasmids		
pHP45-Ω(Cm^r)	Plasmid carrying a Ω(Cm^r) cassette; Ap^r Cm^r	30
pTX419	pBR322 containing 1.3-kb *Hin*dIII-*Eco*RI *epd*$^+$ (*gapB*$^+$) fragment; Ap^r	36
pTX457[c]	pTX419 *epd* (*gapB*)::Ω(Cm^r); Ω(Cm^r) cassette from pHP45-Ω(Cm^r) cloned into the *Cla*I site of pTX419; Ap^r Cm^r	This study

[a] Ap^r and Cm^r, resistance to ampicillin and chloramphenicol, respectively.
[b] Contains point mutation in *gapA* (20).
[c] See Fig. 2 for location of Ω(Cm^r) insertion in *epd* (*gapB*).

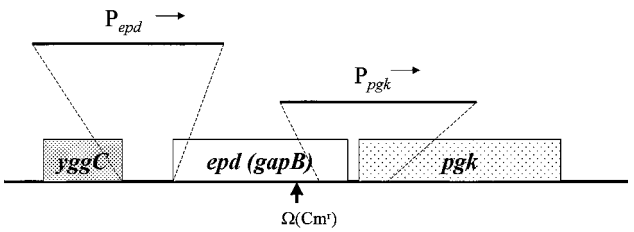

FIG. 2. Structure of the *epd* (*gapB*) gene (drawn to scale) at 66.17 min in the *E. coli* K-12 chromosome (1, 15, 21). *epd* (*gapB*) is surrounded by and oriented in the same direction as *yggC*, whose function is unknown, and *pgk*, which encodes the glycolytic enzyme phosphoglycerate kinase (1). Promoter mapping studies to be published elsewhere locate the promoters for *epd* (*gapB*) (P_{epd}) and *pgk* (P_{pgk}) in the regions indicated by bars. The location of the *epd*::Ω(Cm^r) insertion mutation constructed in this study (Table 1) is indicated. The *epd*::Ω(Cm^r) insertion mutation is upstream from the P_{pgk} promoter region and does not interfere with transcription of the *pgk* gene (data not shown).

tained a 4-bp deletion created during cloning, into the bacterial chromosome by transformation with linearized plasmid DNA (Fig. 2; Table 1) (2, 33). The *epd*::Ω(Cm^r) mutation was crossed into the W3110 and MG1655 genetic backgrounds by generalized transduction (Table 1). Western immunoblotting showed that the Epd enzyme was expressed in the W3110 *epd*$^+$ parent but not in the W3110 *epd*::Ω(Cm^r) mutant (data not shown). The following G3P dehydrogenase-specific activities were obtained in crude extracts (36) of the W3110 *gapA*$^+$ *epd*$^+$, *gapA*$^+$ *epd*::Ω(Cm^r), *gapA1 epd*$^+$, and *gapA1 epd*::Ω(Cm^r) strains: 1,746 ± 53, 1,835 ± 117, 91 ± 4, and 0 nmol per min per mg of protein, respectively. Lack of or low residual G3P dehydrogenase activity in the *gapA epd* or *gapA epd*$^+$ mutant, respectively, agrees with previous results (9).

Growth properties of *gapA*$^+$ *epd*::Ω(Cm^r) mutants. In contrast to the results of Della Seta et al. (9), we observed that colony formation of the *gapA*$^+$ *epd*::Ω(Cm^r) mutant was impaired on plates containing MM [Vogel-Bonner (1 × E) (8) or M63 (27)] supplemented with glycerol plus succinate or Casamino Acids as carbon sources (Table 2). This impaired growth was relieved by the addition of PN (Table 2) or glycolaldehyde (GA), which can be converted to 4PHT by an alternative pathway (14, 37). Growth of the *gapA*$^+$ *epd*::Ω(Cm^r) mutant was not significantly impaired on MM plates containing glucose, acetate, ribose, xylulose, fructose, or gluconic acid or in liquid medium containing glycerol plus succinate or Casamino Acids as carbon sources (Table 2 and data not shown).

We performed high-performance liquid chromatography (HPLC) (31) to confirm that the slower growth of the W3110 *gapA*$^+$ *epd*::Ω(Cm^r) mutant on MM plates containing glycerol plus succinate was correlated with reduced cellular levels of PLP and PMP (Fig. 3; Table 3). Of the six B_6 vitamers, we detected only PLP and PMP in stationary-phase cells washed from plates after 2 or 3 days and suspended and sonicated in cold 5% metaphosphoric acid (31). Consistent with the growth

TABLE 2. Growth deficiencies of *gapA*$^+$ *epd*::Ω(Cm^r) and *gapA1 epd*::Ω(Cm^r) mutants on MM lacking PN[a]

Strain (genotype)	Supplement[b]	Colony areas (mm^2) on MM plates containing the following carbon sources[c]:			
		Glucose	Glycerol + succinate	Casamino Acids	Sodium acetate
TX3470 (W3110 *gapA*$^+$ *epd*$^+$)	None	3.9	1.7	0.07	0.29
	PN	3.8	1.7	0.07	0.28
TX3484 (W3110 *gapA epd*$^+$)	None	0.04	0.14	NT	0.20
	PN	0.03	0.15	NT	0.30
TX3481 (W3110 *gapA*$^+$ *epd*)	None	3.7	0.35	NG	0.07
	PN	3.8	1.7	0.07	0.27
TX3491 (W3110 *gapA epd*)	None	NG	NG	NT	NG
	PN	0.03	0.07	NT	0.13
TX4090 (MG1655 *gapA*$^+$ *epd*$^+$)	None	4.4	1.5	1.1	0.48
	PN	4.5	1.5	1.1	0.49
TX4125 (MG1655 *gapA epd*$^+$)	None	NG	0.79	NT	0.22
	PN	NG	0.79	NT	0.23
TX4092 (MG1655 *gapA*$^+$ *epd*)	None	3.6	0.75	0.18	0.40
	PN	4.5	1.5	1.1	0.43
TX4134 (MG1655 *gapA epd*)	None	NG	NG	NT	NG
	PN	NG	0.79	NT	0.22

[a] Bacteria were grown in LB containing 1% (vol/vol) glycerol, 1% (wt/vol) succinate (disodium hexahydrate), and all appropriate antibiotics with shaking at 37°C for about 24 h. Bacteria were collected by centrifugation and washed twice with (1 × E) minimal salts at 25°C (8). Washed cells were diluted by 10^6 in (1 × E) minimal salts, and 150 μl of the cell suspensions was spread onto the (1 × E) plates containing the indicated supplements and carbon sources (no antibiotics). The plates were incubated at 37°C.

[b] 2 μM PN was added as indicated. Addition of 0.1 mM GA gave the same results as PN addition (data not shown).

[c] For each medium, areas were calculated from the diameters of at least 50 well-separated colonies. Experimental errors of colony areas were less than 15%. Totals of 0.4% (wt/vol) glucose, 0.2% (vol/vol) glycerol plus 0.2% (wt/vol) succinate, 0.5% (wt/vol) Difco vitamin assay Casamino Acids, and 0.4% (wt/vol) sodium acetate were included in the plates as carbon sources. Diameters were measured after 24 h (eight strains on plates containing Casamino Acids), 48 h (all strains on plates containing glucose or glycerol plus succinate; MG1655 strains on plates containing sodium acetate), or 72 h (W3110 strains on plates containing sodium acetate). NT, not tested; NG, no growth detected.

characteristics (Table 2), the W3110 *gapA*$^+$ *epd*::Ω(Cm^r) mutant contained only 64% of the PLP and PMP compared to the W3110 *gapA*$^+$ *epd*$^+$ parent, where most of the difference was a decrease in the amount of PMP (Table 3). Likewise, the W3110 *gapA1 epd*$^+$ mutant contained only about 60% as much PMP as the parent; however, both strains contained equal amounts of PLP (Table 3). Finally, by assuming about 6 μl of water per mg of protein (28), we calculate that the stationary-phase W3110 *gapA*$^+$ *gapB*$^+$ parent contained about 76 and 36 μM PLP and PMP, respectively. These amounts are somewhat greater than the 40 μM combined intracellular concentration of PLP and PMP reported previously for *E. coli* K-12 (11). However, unlike this other report (11), we failed to detect appreciable B_6 vitamers excreted into the growth medium.

FIG. 3. HPLC chromatograms of B_6 vitamers extracted from strains TX3470 (*gapA*$^+$ *epd*$^+$ parent) (top), TX3481 [(*gapA*$^+$ *epd*::Ω(Cm^r)] (middle), and TX3484 (*gapA1 epd*$^+$) (bottom). Strains were grown on plates as described in footnote *a* to Table 3, and B_6 vitamers were partially purified by metaphosphoric acid and dichloromethane extractions and were resolved by reverse-phase, ion-pair HPLC on an Ultremex 3 C_{18} column (150 by 4.6 mm) (Phenomenex, Inc.) fitted with a guard column (30 by 4.6 mm) at 0.5 ml per min as described elsewhere (31). The tracings show the fluorescence intensity (excitation at 328 nm; emission at 393 nm) of postcolumn adducts between the B_6 vitamers and sodium bisulfite (31). The elution positions and fluorescence yields of the six B_6 vitamers (PLP, PMP, PNP, PL, PM, PN) were determined by using pure compounds as standards (data not shown). Only PLP and PMP (top panel) were detected in extracts of *E. coli* K-12. Two peaks that are not B_6 vitamers were routinely detected (top panel) and were used along with protein amounts of the cells before extraction to normalize PLP and PMP amounts in different preparations. Amounts of PLP and PMP were determined (Table 3) by integration of peaks and comparison of areas with those of the PLP and PMP standard curves, which were linear over this range of concentrations. The fluorescence yield of the PMP-bisulfite adduct was approximately 5.6-fold greater than that of the PLP-bisulfite adduct (data not shown). This difference accounts for the fact that PLP peaks, which are apparently smaller than PMP peaks, actually correspond to more PLP than PMP (Fig. 3; Table 3).

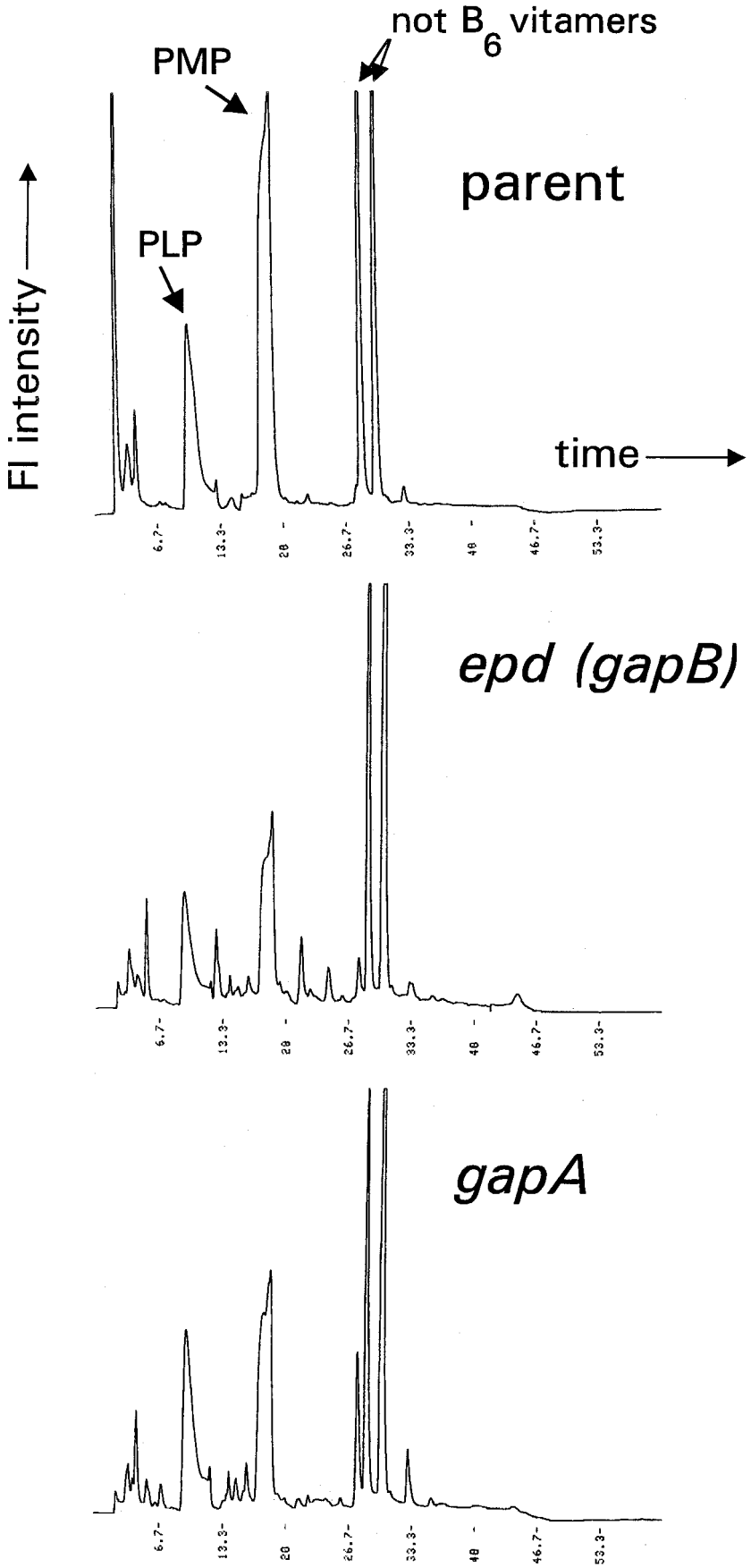
not B6 vitamers
PMP
parent
PLP
Fl intensity
time
6.7-
13.3-
20 -
26.7-
33.3-
40 -
46.7-
53.3-
epd (gapB)
gapA

TABLE 3. Amounts of B_6 vitamers in *gapA*$^+$ *epd*$^+$, *gapA-1 epd*$^+$, and *gapA*$^+$ *epd*::Ω(Cm^r) strains

Strain	Amt (pmol) of B_6 vitamer per mg of protein (%)[a]		
	PLP	PMP	PLP + PMP (%)
TX3470 (W3110 *gapA*$^+$ *epd*$^+$)	455 ± 99 (67)	218 ± 8 (33)	673 (≡100)
TX3484 (W3110 *gapA1 epd*$^+$)	451 ± 18 (67)	126 ± 23 (19)	577 (86)
TX3481 [W3110 *gapA*$^+$ *epd*::Ω (Cm^r)]	329 ± 74 (49)	103 ± 6 (15)	432 (64)

[a] Bacteria were grown on plates containing (1 × E) minimal salts, 1% (vol/vol) glycerol, and 1% (wt/vol) succinate at 37°C for 48 h (TX3470 and TX3481) or 72 h (TX3484) (see Table 2). Cells were washed from plates with ice-cold (1 × E) minimal salts, and suspensions were adjusted to a turbidity of 100 Klett (660 nm) Units. Cells from 50 ml of suspension were collected by centrifugation, washed with (1 × E) minimal salts, and centrifuged again, where all steps were performed at 4°C. Cells were suspended in 2.5 ml of ice-cold metaphosphoric acid under yellow lights, and B_6 vitamers were extracted and resolved by reverse-phase, ion-pair HPLC (Fig. 3) (31). Protein amounts were determined by the D_c (Lowry) protein assay (Bio-Rad Laboratories) with bovine serum albumin as a standard in cell suspensions that were brought to 0.5% (wt/vol) in sodium dodecyl sulfate and boiled for 10 min.

Thus, the impaired growth of the *gapA*$^+$ *epd*::Ω(Cm^r) mutant was correlated with a 40% reduction in the amounts of cellular PLP and PMP.

PN auxotrophy of *gapA epd* double mutants. Most importantly, we found that the *gapA1 epd*::Ω(Cm^r) double mutant was auxotrophic for PN on MM containing glycerol plus succinate (Table 2). After 2 days of incubation, we could not detect growth of the *gapA1 epd*::Ω(Cm^r) double mutant, and after 3 days, we detected very small colonies which may have arisen by leakiness of the *gapA1* point mutation. Growth was restored by the addition of PN or GA (Table 2). Unexpectedly, *gapA1* mutants of MG1655 and W3110 grew on MM containing 0.4% sodium acetate as the carbon source (Table 2), whereas it was reported that the original DF220 *gapA1* mutant in the K10 genetic background failed to grow on MM containing acetate (20). Nonetheless, the MG1655 and W3110 *gapA1 gapB*::Ω (Cm^r) double mutants were again auxotrophic for PN on MM containing acetate (Table 2).

We confirmed the conclusion that *gapA epd* double mutants are auxotrophic for PN in one other way. We simply transduced the *epd*::Ω(Cm^r) mutation into strain DF221 that contained the *gapA2* nonsense allele (20), which is different from the *gapA1* point mutation from strain DF220 used in the experiments described above (Table 1). Colonies of the resulting *gapA2 epd*::Ω(Cm^r) double mutant TX4187 appeared on MM plates containing glycerol plus succinate and PN in 2 to 3 days; however, no colonies appeared after 5 days when PN was omitted from the growth medium (data not shown). DF221 (*gapA-2*) failed to grow on MM containing glucose after 3 days at 37°C, but it did grow on MM containing acetate (data not shown). These growth properties might be explained by slight leakiness of the *gapA1* and *gapA2* mutations such that there was sufficient gluconeogensis to allow growth on acetate but insufficient glycolysis to support growth on glucose. Finally, it could be argued that a mutation in another gene that cotransduces with the *gapA1* point mutation caused the PN auxotrophy (Table 2). To rule out this hypothesis, we analyzed 20 independent spontaneous mutants of TX3491 and TX4134 [*gapA1 epd*::Ω(Cm^r)] that grew rapidly as did the *gapA*$^+$ *epd*::Ω(Cm^r) strain on MM containing 0.4% (wt/vol) glucose. For all 20 mutants, reversion or suppression of the *gapA1* mutation not only allowed growth on glucose medium but also alleviated the PN requirement.

Summary. Together, our data show that the GapA and Epd dehydrogenases are required for de novo PLP biosynthesis, and this involvement supports the pathway depicted in Fig. 1. The contribution of Epd dehydrogenase to 4PE synthesis seems to vary and becomes more significant in colonies growing on certain nonglycolytic carbon sources (Tables 2 and 3). We do not understand why the growth of *gapA*$^+$ *epd* mutants is impaired on solid but not in liquid media (see above; Tables 2 and 3). In cells grown on glycolytic carbon sources, such as glucose, we observed that the specific activity of G3P dehydrogenase increased at least twofold in crude extracts compared to cells grown in MM containing glycerol plus succinate or acetate (data not shown). Therefore, it seems likely that the GapA enzyme alone is sufficient to carry out PLP biosynthesis in cells growing on glycolytic carbon sources. The involvement of the GapA enzyme in PLP biosynthesis is consistent with low levels of E4P dehydrogenase detected for some GapA dehydrogenases (5), and the enzymatic redundancy of GapA and Epd would explain why mutants deficient in this first step of branch 1 of PLP biosynthesis were never isolated (10, 23). Using purified enzymes, we did not detect feedback inhibition of the Epd E4P or GapA G3P dehydrogenase activities by 4PHT (data not shown). This finding is consistent with the idea that PLP biosynthesis responds to the carbon source and overall metabolic state instead of to the amounts of pathway end products (26).

We can only speculate as to why we obtained results completely different from those of Della Seta et al. (9). In their experiments, the parent and mutant strains seem to have been spread onto plates containing different combinations of antibiotics corresponding to the insertions in their *gapA* and *epd* mutants (9). However, it is not immediately clear why antibiotic addition would bypass the requirement for GapA and Epd in PLP biosynthesis. Likewise, it is difficult to see how polarity of the *gapA* and *epd* insertion mutations, if any, could bypass the need for an E4P dehydrogenase in PLP biosynthesis. In their experiments, no control was mentioned to test media for traces of B_6 vitamers that would allow growth of the *gapA epd* double mutant. Finally, it seems possible that their *gapA epd* (*gapB*) double mutant may have acquired a suppressor mutation that allowed growth without supplementation with B_6 vitamers. Another partial homolog of *gapA*, called *gapC*, is present in *E. coli* K-12, but *gapC* is not thought to encode a functional dehydrogenase (5, 17).

We thank D. G. Fraenkel, H. M. Krisch, C. A. Gross, A. J. Clark, and C. Yanofsky for the strains and plasmids used in this study and members of this laboratory for helpful discussions and critical comments.

This work was supported by Public Health Services grant RO1-GM37561 from the National Institute of General Medical Sciences.

Yong Yang and Genshi Zhao contributed equally to this work.

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