JOURNAL OF BACTERIOLOGY, June 1997, p. 3989–3996 Vol. 179, No. 12
0021-9193/97/$04.00+0

Mutations in *sdh* (Succinate Dehydrogenase Genes) Alter the Thiamine Requirement of *Salmonella typhimurium*

JODI L. ENOS-BERLAGE AND DIANA M. DOWNS*

Department of Bacteriology, University of Wisconsin-Madison, Madison, Wisconsin 53706

Received 12 March 1997/Accepted 11 April 1997

Mutants lacking the first enzyme in de novo purine synthesis (PurF) can synthesize thiamine if increased levels of pantothenate are present in the culture medium (J. L. Enos-Berlage and D. M. Downs, J. Bacteriol. 178:1476–1479, 1996). Derivatives of *purF* mutants that no longer required pantothenate for thiamine-independent growth were isolated. Analysis of these mutants demonstrated that they were defective in succinate dehydrogenase (Sdh), an enzyme of the tricarboxylic acid cycle. Results of phenotypic analyses suggested that a defect in Sdh decreased the thiamine requirement of *Salmonella typhimurium*. This reduced requirement correlated with levels of succinyl-coenzyme A (succinyl-CoA), which is synthesized in a thiamine pyrophosphate-dependent reaction. The effect of succinyl-CoA on thiamine metabolism was distinct from the role of pantothenate in thiamine synthesis.

The goal of our research is to investigate metabolic connections that extend beyond linear biochemical pathways. Thiamine synthesis in *Salmonella typhimurium* serves as a model system for studying these aspects of metabolism. Several unexpected relationships exist between thiamine synthesis and other metabolic pathways in the cell, including, as reported to date, the pentose phosphate pathway, carbon catabolism, and pantothenate biosynthesis (10, 12, 13, 26, 27). In addition, because thiamine is required in small amounts, growth in the absence of thiamine provides a very sensitive assay for endogenous synthesis. Intuitively, pathways with high carbon flux may contribute precursors to low-flux pathways without significant depletion of their intermediates. This may provide the cell with detectable flexibility in supplying its low-level requirements.

Thiamine pyrophosphate (TPP), the biologically active form of thiamine, is a cofactor for metabolic reactions involving the transfer of C_2 units, including reactions catalyzed by pyruvate dehydrogenase, pyruvate decarboxylase, and α-ketoglutarate dehydrogenase. TPP is formed by the joining and subsequent phosphorylation of two independently synthesized precursors, 4-methyl-5(β-hydroxyethyl) thiazole phosphate (THZ-P) and 4-amino-5-hydroxymethyl-2-methylpyrimidine-pyrophosphate (HMP-PP) (Fig. 1A). Although analysis of the biosynthetic pathway for THZ-P is in the early stages, labeling studies have indicated that this moiety is derived from cysteine, tyrosine, and 1-deoxy-D-xylulose (7, 9, 15).

Our work is currently focused on the routes of HMP synthesis and their utilization under different growth conditions. Three genetically separable routes have been found to contribute to the synthesis of HMP in *S. typhimurium* (Fig. 1A). The first five steps of the well-characterized purine pathway form 5-aminoimidazole ribotide, which can then be converted to HMP by putative Thi gene products (14, 26). A second pathway to HMP, the alternative pyrimidine biosynthetic (APB) pathway, bypasses the requirement for purine biosynthetic enzymes under some growth conditions (10, 11, 27, 36). Finally, HMP can be generated via the pantothenate-dependent pyrimidine (PDP) pathway. It is important to emphasize that while for simplicity we refer to these routes as pathways, it is possible that some of the metabolites involved are products of side reactions of known enzymes and are not formed by enzymes dedicated to thiamine synthesis.

The PDP pathway appears to bypass function of the PurF enzyme, amidophosphoribosyl transferase (EC 2.4.2.14), suggesting that the product of this pathway is phosphoribosylamine (PRA) (12). Function of the PDP pathway required increased levels of exogenous pantothenate and a source of ribose-5-P, either supplied exogenously or generated by a functional oxidative pentose phosphate pathway (12, 13). The role of pantothenate in thiamine synthesis has remained unclear since its only demonstrated role is to function as a precursor to coenzyme A (CoA) (19).

We report here work initiated to define the role of pantothenate in thiamine synthesis. Mutations that allowed a *purF* mutant to grow independently of pantothenate or thiamine were isolated. Characterization of these mutations demonstrated that they were lesions in *sdh*, which encodes succinate dehydrogenase (Sdh) (Fig. 1B). Evidence is presented that suggests that a block in Sdh causes an effect on thiamine metabolism through an alteration in the succinyl-CoA pool; this effect is distinct from the role of pantothenate in thiamine synthesis. A model for how this alteration allows pantothenate- and thiamine-independent growth is discussed.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. All strains used in this study are derived from *S. typhimurium* LT2 and are listed with their respective genotypes in Table 1. The NCE medium of Berkowitz et al. (2) supplemented with 1 mM MgSO4 was used as minimal medium. The following carbon sources were added at the concentrations indicated: glucose, 11 mM; citrate, 11 mM; glycerol, 55 mM. Difco nutrient broth (8 g/liter) with NaCl (5 g/liter) added was used as rich medium. Difco BiTek agar was added to a final concentration of 1.5% for solid medium. When present in the culture media, and unless otherwise stated, the compounds were used at the following final concentrations: adenine, 0.4 mM; thiamine, 0.5 μM; pantothenate, 100 μM; lysine, 0.25 mM; methionine, 0.3 mM; diaminopimelic acid, 0.1 mM; succinate, 4 mM. The final concentrations of the antibiotics in rich or minimal medium were as follows: tetracycline, 20 or 10 μg/ml, respectively; kanamycin, 50 or 125 μg/ml, respectively; ampicillin, 30 or 15 μg/ml, respectively. Unless otherwise stated, all chemicals and enzymes were purchased from Sigma Chemical Co., St. Louis, Mo.

Genetic techniques. (i) Transduction methods. The high-frequency general transducing mutant of bacteriophage P22 (HT105/1, *int-201*) (29) was used in all

* Corresponding author. Mailing address: University of Wisconsin-Madison, 1550 Linden Dr., Madison, WI 53706. Phone: (608) 265-4630. Fax: (608) 262-9685. E-mail: Downs@vms2.macc.wisc.edu.

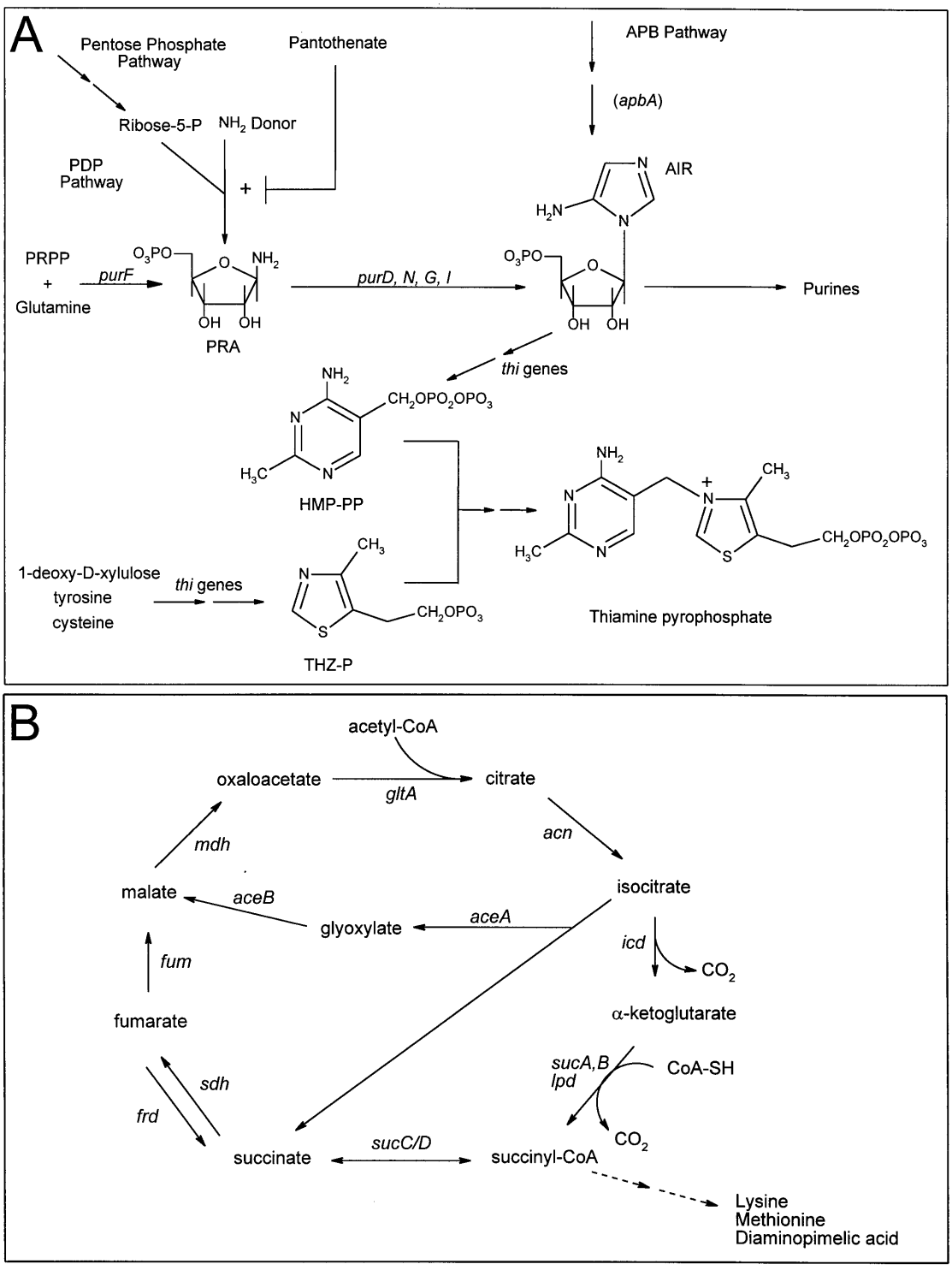

FIG. 1. Pathway schematics. (A) Biosynthetic pathways involved in thiamine synthesis. Genes whose products are required for selected reactions are indicated next to the relevant arrows. Aside from the purine pathway, specific steps in the synthesis of HMP-PP and THZ-P are unknown. The positive role of pantothenate in the PDP pathway is indicated (12), along with the proposed precursor, ribose-5-P, and the product, phosphoribosylamine (13). The designation of 5-aminoimidazole ribotide as the product of the APB pathway is based on genetic data (27). Abbreviations: PRPP, phosphoribosylpyrophosphate; PRA, phosphoribosylamine; AIR, 5-aminoimidazole ribotide. (B) The TCA cycle and glyoxylate shunt (6). Hashed arrows represent multiple steps and indicate the role of succinyl-CoA in the synthesis of essential compounds.

TABLE 1. Bacterial strains

Strain	Genotype	Source[a]
LT2	Wild type	Laboratory strain
JE1212	*metE205 ara-9 sucCD211*::MudJ[b]	J. C. Escalante-Semerena
JRGS6si	*sucA*	SGSC[d]
DM95	*thiC885*::MudJ	
DM269	*thiI887*::Tn*10*d(Tc)[c]	
DM384	*purF2085 apbA1*::MudJ	
DM587	*purF2085 apbA7*::Tn*10*d(Tc)	
DM588	*purF2085 apbA1*::MudJ	
DM707	*purF2085 zbg-8063*::Tn*10*d8063::Tn*10*d(Tc)	
DM708	*purF2085 zbg-8063*::Tn*10*d(Tc) *sdh-201*	
DM711	*purF2085 zbg-8081*::Tn*10*d(Tc) *sdh-202*	
DM712	*purF2085 zbg-8081*::Tn*10*d(Tc)	
DM754	*purF2085 sdhC203*::Tn*10*d(Tc)	
DM1254	*purF2085 apbA1*::MudJ *zbg-8063*::Tn*10*d (Tc) *sdh-201*	
DM1936	*purF2085*	
DM2357	*zbg-8063*::Tn*10*d(Tc) *sdh-201*	
DM2358	*zbg-8063*::Tn*10*d(Tc)	
DM2359	*zbg-8081*::Tn*10*d(Tc) *sdh-202*	
DM2360	*zbg-8081*::Tn*10*d(Tc)	
DM2361	*sdhC203*::Tn*10*d(Tc)	
DM2370	*purF2085 gltA1182*::MudJ	
DM2371	*purF2085 gltA1182*::MudJ *sdhC203*:: Tn*10*d(Tc)	
DM2378	*purF2085 apbA1*::MudJ *zbg-8063*::Tn*10*d (Tc) *sdh-201*	
DM2398	*purF2085 sucCD211*::MudJ	
DM2404	*purF2085 sucA*	
DM2408	*purF2085 sucA sucCD211*::MudJ	

[a] Unless otherwise indicated, strains were constructed for this study.
[b] MudJ is used throughout the text to refer to the Mud I1734 transposon (3).
[c] Tn*10*d(Tc) refers to the transposition-defective mini-Tn*10* (Tn*10* Δ-16 Δ-17) (32).
[d] SGSC, Salmonella Genetic Stock Center, Calgary, Alberta, Canada.

TABLE 2. Plasmids

Plasmid	Parent plasmid	Phenotype	Size of insert (kb)	Source[a]
pSDH1	pBR328	Ap^r Tc^s Sdh^+	53.5	Plasmid library
pFRD1	pBR328	Ap^r Tc^s Sdh^+	7.85	Plasmid library
pFRD1a	pSU19	Cm^r Sdh^-	1.4	*Eco*RI fragment from pFRD1

[a] The plasmid library is that of C. Miller, University of Illinois, Urbana-Champaign.

transductional crosses. Protocols for transductional crosses, freeing mutants of contaminating phage, and assessing phage sensitivity have been described (11).

(ii) Isolation of *sdh* mutants. A P22 lysate grown on a pool of >70,000 cells containing random Tn*10*d(Tc) insertions was mutagenized with hydroxylamine as described previously (8, 18) and used to transduce DM384 (*purF2085 apbA1*::MudJ) or DM1936 (*purF2085*) to tetracycline resistance (Tc^r) on nutrient agar plates. The Tc^r transductants were screened for growth on glucose medium supplemented only with adenine and tetracycline. The mutations identified in the *purF* background were transduced into DM588 (*purF2085 apbA1*::MudJ) by selecting Tc^r to confirm that thiamine-independent growth was not dependent on ApbA.

(iii) Construction of *purF sucA sucCD* mutants. The *purF sucA sucCD* triple mutants were constructed as follows. The *sucA* mutation (20) was transduced into DM2370 (*purF2085 gltA*::MudJ) based on its linkage to *gltA*, resulting in strain DM2404 (*purF2085 sucA*). The *sucCD-211*::MudJ insertion was transduced into DM2404 by selecting Km^r. The Km^r transductants that retained the *sucA* mutation were identified as those that required lysine, methionine, and diaminopimelic acid (DAP) for growth.

***Xba*I mapping analysis.** The Tn*10*d(Tc) insertions in strains DM708 [*zbg-8063*::Tn*10*d(Tc)], DM711 [*zbg-8081*::Tn*10*d(Tc)], and DM754 [*sdhC203*::Tn*10*d (Tc)] were mapped by use of *Xba*I analysis as described by Liu and Sanderson (22). This analysis resulted in the splitting of band A into two new fragments, placing the Tn*10*d(Tc) insertions in strains DM708, DM711, and DM754 between 12 and 29 centisomes on the *S. typhimurium* chromosome.

Phenotypic analysis. Procedures for testing nutritional requirements on solid medium by use of agar overlays and on liquid medium by use of growth curves have been described (27). Specific growth rate was determined as $\mu = \ln(X/X_O)/T$, where X is the A_{650} during the linear portion of the growth curve and T is time. Routinely, the A_{650} at time zero was between 0.02 and 0.04. When strains were subcultured, 0.15 ml of a full-density culture (or equivalent number of cells) was used to inoculate 5 ml of fresh medium.

Isolation of complementing clones. Plasmids used in this study are listed in Table 2. Clones complementing the Sdh^- phenotype were isolated from a plasmid library of *S. typhimurium* LT2 chromosomal DNA digested with *Sau*3A and cloned into the *Bam*HI site of the *tetA* gene of vector pBR328 (Ap^r Cm^r; 4.9 kb; C. Miller, University of Illinois, Urbana-Champaign). Plasmids were introduced into DM1254 [*purF2085 apbA1*::MudJ *zbg-8063*::Tn*10*d(Tc) *sdh-201*] by P22 transduction, and Ap^r transductants able to grow with citrate as the sole carbon source were identified. Two complementing plasmids, pSDH1 (3.5-kb insert) and pFRD1 (7.8-kb insert), were obtained. Digestion of pFRD1 with *Eco*RI yielded a 1.4-kb fragment that was subcloned into pSU19, creating plasmid pFRD1a.

Molecular biology techniques. Standard methods were used for DNA restriction endonuclease digestion and ligation. Restriction enzymes and ligase were purchased from Promega (Madison, Wis.). Plasmid DNA was isolated with the QIAprep spin plasmid kit purchased from Qiagen (Chatsworth, Calif.). Plasmid DNA was transferred between strains by electroporation with a Bio-Rad (Richmond, Calif.) *E. coli* Pulser as suggested by the manufacturer.

PCR amplification. Primers specific to the ends of the MudJ and Tn*10*d(Tc) insertions (5′-GCTTTCGCGTTTTTCGTG-3′ and 5′-GACAAGATGTGTATC CACCTTAAC-3′, respectively) were used to PCR amplify the DNA between the *gltA1182*::MudJ and *sdhC203*::Tn*10*d(Tc) insertions in DM2371 as described previously (34). Amplification was performed with Vent (exo^-) polymerase (New England Biolabs, Inc., Beverly, Mass.) in a Thermolyne Temp-Tronic thermocycler. PCR conditions were denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min. This resulted in amplification of an 870-bp fragment, one end of which was sequenced with the primer to the Tn*10*d(Tc) insertion.

Sequencing. Dideoxy sequencing (28) was performed with a Sequitherm cycle sequencing kit (Epicentre Technologies, Madison, Wis.) and [^{35}S]dATP with a specific activity of 1,000 to 1,500 Ci/mmol (Dupont, Beverly, Mass.). Primers that hybridized to either side of the *Bam*HI site in pBR328 were used to sequence the ends of the insert in pSDH. The −40 and reverse primers for M13mp19 (New England BioLabs) were used to sequence the ends of the 1.4-kb subclone in pFRD1a.

Succinate dehydrogenase assays. (i) Preparation of extracts. A 200-ml culture was grown in NCE glycerol medium to the late log phase (100 Klett units), harvested by centrifugation (10,000 × *g*, 10 min), and washed twice with cold 50 mM potassium phosphate buffer (pH 7.5). The cell pellets were resuspended in 1.5 ml of the same buffer plus 1 mM phenylmethylsulfonyl fluoride (Aldrich Chemical Co., Inc., Milwaukee, Wis.) and disrupted by sonication. Cell debris was removed by centrifugation at 10,000 × *g* for 30 min at 4°C.

(ii) Sdh assays. Succinate dehydrogenase activity was measured by the method of Spencer and Guest (31). The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.5), 3 mM KCN, 0.23 mM 2,6-dichlorophenol indophenol (DCPIP), 2.18 mM *N*-methylphenazonium methosulfate, and crude extract (up to 0.2 mg of protein) in a final volume of 1 ml. Sample and reference cuvettes were allowed to equilibrate for 1 to 2 min, and the reaction was initiated by the addition of 10 mM succinate. The reduction of DCPIP (molar extinction coefficient, 21×10^3 cm^{-1}) was monitored at 600 nm.

Computer analysis. BLAST (Basic Local Alignment Search Tool) was used to search for homologous DNA and protein sequences from the GenBank and SwissProt databases (1).

RESULTS

Initial observations. Both *purF* and *purF apbA* mutants can grow on glucose adenine medium supplemented with either thiamine or pantothenate (13). To identify the role of pantothenate in thiamine synthesis, derivatives of these mutants that could grow in the absence of thiamine or pantothenate were isolated (see Materials and Methods). To ensure that the APB pathway was not responsible for this thiamine-independent growth, we demanded that an *apbA* mutation (11) not eliminate growth of these mutants on glucose adenine medium. After screening 8,000 transductants, three mutants with the desired phenotype were identified, two in a *purF* background and one in a *purF apbA* background. Two of the three mutants contained point mutations, 40 and 46% linked to the selected

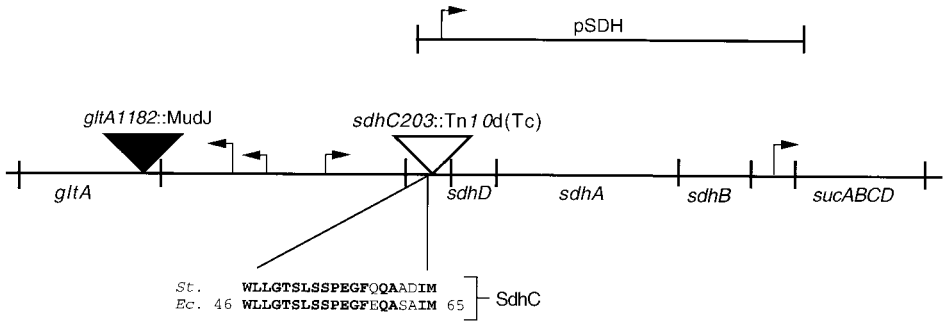

FIG. 2. Location of the *sdhC203*::Tn*10*d(Tc) insertion. A schematic representation of the TCA cycle gene cluster in *E. coli* (Ec.) and *S. typhimurium* (St.) located at 17 centisomes is shown. The *sdhC203*::Tn*10*d(Tc) insertion was mapped within the *sdhC* gene based on sequence homology with *E. coli*. Sequences flanking the insertion were determined, and the predicted amino acids are shown; conserved amino acids between the two organisms are in boldface type, and numbers represent amino acids in *E. coli* SdhC protein. The DNA fragment carried by pSDH1 is shown. Tall arrows indicate major promoters; short arrows represent minor promoters.

Tn*10*d(Tc) insertion (*sdh-201* and *sdh-202*, respectively). In the third case, the mutant phenotype was 100% linked to the Tn*10*d(Tc) insertion, and thus the insertion was presumed to be causative [*sdh-203*::Tn*10*d(Tc)]. The identification of an insertion mutation was unexpected since the desired phenotype was a gain of function, i.e., a gained ability to make thiamine without exogenous pantothenate.

Lesions in *sdh* allow pantothenate- and thiamine-independent growth of a *purF* mutant. Further analysis determined that the three mutations mentioned above were lesions in *sdh*, which encodes succinate dehydrogenase, an enzyme of the tricarboxylic acid (TCA) cycle (Fig. 1B). Identification of these mutations as alleles of *sdh* was based on the following results. (i) Mutant phenotypes were complemented by either the *sdh* or *frd* genes. We determined that the three mutants described above were unable to use citrate as the sole carbon source (Cit^-). Two clones that complemented the Cit^- phenotype of DM1254 (*purF apbA sdh*) were isolated. Both plasmids pSDH1 and pFRD1 restored growth of this strain on citrate as the sole carbon source and simultaneously restored the need for pantothenate in thiamine synthesis. Complementation with pFRD1, however, was significantly weaker than that with pSDH1 (data not shown). Sequence analysis determined that pSDH1 contained the *sdhDAB* genes in addition to one of the promoters for this operon (Fig. 2) (35). Interestingly, although pSDH1 complemented the Cit^- phenotype of both *sdh* point mutants, DM708 and DM711, it failed to complement the insertion mutant, DM754. The data described below that demonstrates that DM754 contained a Tn*10*d(Tc) insertion in *sdhC* clarified this result, based on the known operon structure of these genes (Fig. 2). Sequence analysis of pFRD1a determined that pFRD1 contained the *frd* operon (data not shown). The *frd* genes have been reported to complement *sdh* mutants when present in multicopy (17) because they encode fumarate reductase, an enzyme that catalyzes the reverse of the Sdh reaction.

(ii) The respective mutants were defective in Sdh activity. Strains DM2357, DM2359, and DM2361, containing the three mutations described above, were assayed for Sdh activity as described in Materials and Methods. The results of these assays, shown in Table 3, revealed that the Sdh activity in each of these strains was at least 15-fold lower than that of the isogenic sdh^+ strain.

(iii) The respective mutations mapped to the *sdh* gene. The Tn*10*d(Tc) insertions in the three mutants, DM708, DM711, and DM754, were mapped between 12 and 29 centisomes on the *S. typhimurium* chromosome by use of *Xba*I analysis (22). Transductional crosses with known genetic markers in this region placed the three insertions at 17 centisomes, linked to a *sucCD211*::MudJ insertion (19, 20, and 32% linked, respectively). The *sdhC203*::Tn*10*d(Tc) insertion was mapped precisely by sequencing a PCR-amplified segment of DNA between this insertion and the *gltA1182*::MudJ insertion (Fig. 2). Sequence analysis of this fragment with a Tn*10* primer determined that the Tn*10*d(Tc) insertion was within the *sdhC* gene as indicated in Fig. 2.

The effect of an Sdh block is reduced by an *apbA* mutation. The stimulatory effect of an *sdh* mutation on thiamine-independent growth in a *purF* and a *purF apbA* genetic background is shown in Fig. 3A and B, respectively. Strains containing the *sdh* mutation (DM708 and DM2378) grew in the absence of pantothenate or thiamine, i.e., on medium supplemented only with adenine. It was clear from the growth of these strains, however, that an *sdh* mutation did not completely eliminate the need for exogenous thiamine in the medium, particularly in the *apbA* derivative. A possible explanation for this growth pattern was that the Sdh block was reducing the amount of thiamine required by the cell, such that the intracellular reserves of TPP accumulated during growth on rich medium (nutrient broth) could sustain a longer period of growth. To address whether the growth of the strains containing *sdh* mutations was due to a decreased thiamine requirement or increased thiamine synthesis, we subcultured these strains into identical media. Both DM708 (*purF sdh*) and DM2378 (*purF apbA sdh*) exhibited greatly reduced thiamine-independent growth after subculturing (Fig. 3C and D). DM708 grew slightly in the absence of

TABLE 3. Mutations that allow thiamine-independent growth of a *purF* mutant reduce succinate dehydrogenase activity[a]

Strain	Genotype	SDH activity (nmol/min/mg)
DM2358	*zbg-8063*::Tn*10*d(Tc)	31.9
DM2357	*zbg-8063*::Tn*10*d(Tc) *sdh-201*	1.8
DM2360	*zbg-8081*::Tn*10*d(Tc)	36.3
DM2359	*zbg-8081*::Tn*10*d(Tc) *sdh-202*	1.0
LT2	Wild type	33.1
DM2361	*sdh203*::Tn*10*d(Tc)	1.9

[a] Strains were grown in minimal medium containing glycerol as the sole carbon source to the late log phase (100 Klett units) and harvested by centrifugation. Crude extracts were prepared by sonication, and Sdh activity was determined by measuring succinate-dependent DCPIP reduction as described in Materials and Methods.

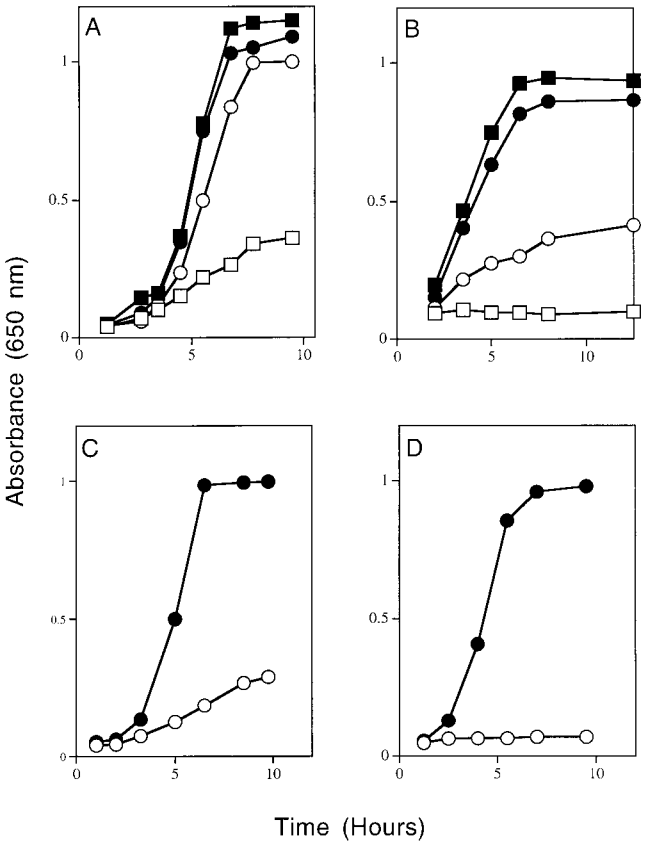

FIG. 3. Effect of an *sdh* mutation on thiamine-independent growth in *purF* and *purF apbA* genetic backgrounds. Strains were grown at 37°C as described in Materials and Methods, and growth curves were obtained. Glucose minimal medium was supplemented with 0.4 mM adenine (open symbols) or 0.4 mM adenine–50 μM thiamine (filled symbols). (A) Growth of DM1936 (*purF2085*) (squares) of DM708 [*purF2085 zbg-8063*::Tn*10*d(Tc) *sdh-201*] (circles); (B) growth of DM587 [*purF2085 apbA7*::Tn*10*d(Tc)] (squares) and of DM2378 [*purF2085 apbA1*::MudJ *zbg-8063*::Tn*10*d(Tc) *sdh-201*] (circles); (C) growth of DM708 [*purF2085 zbg-8063*::Tn*10*d(Tc) *sdh-201*] after subculturing from the culture grown in glucose adenine medium shown in panel A; (D) growth of DM2378 [*purF2085 apbA1*::MudJ *zbg-8063*::Tn*10*d(Tc) *sdh-201*] after subculturing from the culture grown in glucose adenine medium shown in panel B.

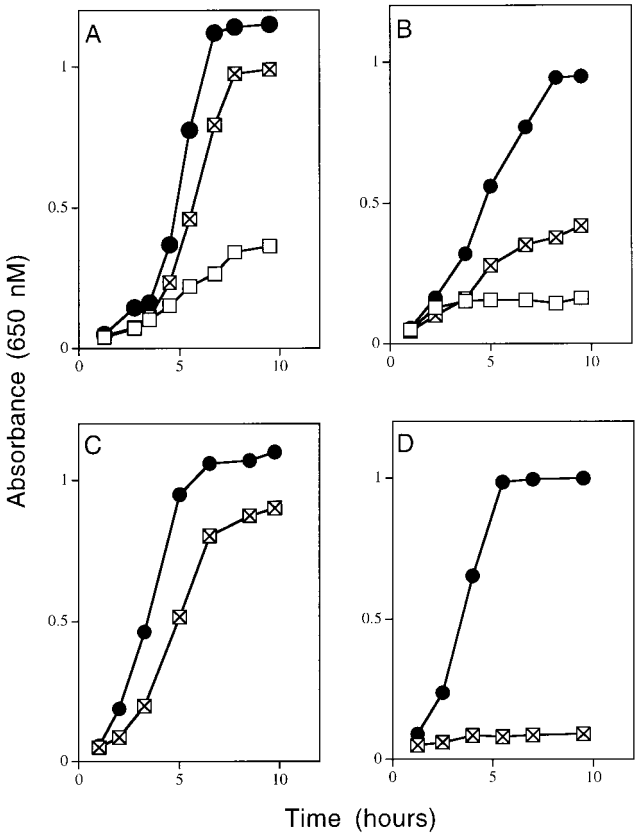

FIG. 4. Effect of succinate on thiamine-independent growth of *purF* and *purF apbA* mutants. Strains were grown at 37°C as described in Materials and Methods, and growth curves were obtained. Glucose minimal medium was supplemented with 0.4 mM adenine (□), 0.4 mM adenine–4 mM succinate (⊠), or 0.4 mM adenine–50 μM thiamine (●). (A) Growth of DM1936 (*purF2085*); (B) growth of DM587 [*purF2085 apbA7*::Tn*10*d(Tc)]; (C and D) growth of DM1936 (*purF2085*) and DM587 [*purF2085 apbA7*::Tn*10*d(Tc)] after being subcultured from the cultures grown in glucose adenine succinate medium shown in panels A and B, respectively.

thiamine (Fig. 3C), but further subculturing eliminated this growth (data not shown). These results suggest that the effect of an Sdh block was at least partially due to a decrease in the cellular thiamine requirement. The more severe phenotype of the *apbA* derivative (DM2378) could be explained if this strain required more thiamine because of loss of residual thiamine synthesis from the APB pathway under these conditions.

The results shown in Fig. 3 also indicate that although the *sdh* mutations were isolated to mimic the effect of pantothenate, their effect was distinct, since both *purF* and *purF apbA* mutants grew continually when pantothenate was added to the medium in place of thiamine (data not shown). This distinction between the effects of exogenous pantothenate and *sdh* mutations on thiamine synthesis was also observed in other experiments (see below).

Although the data described above were consistent with an Sdh block decreasing the amount of thiamine required for growth, the reason for this effect was not clear nor was it clear that this model accounted for all of our results. Subsequent experiments were performed to address these questions.

Exogenous succinate mimics a block in Sdh. Since loss of the Sdh enzyme had produced a gain-of-function phenotype, i.e., increased growth in the absence of thiamine, we pursued the idea that this effect was mediated by a metabolite of the Sdh reaction. A mutant blocked in Sdh might be expected to accumulate succinate (Fig. 1B). We found that exogenous succinate allowed both a *purF* (DM1936) and a *purF apbA* mutant (DM587) to grow in the absence of pantothenate or thiamine. The growth of these strains on glucose adenine medium supplemented with succinate is represented in Fig. 4A and B, respectively. This growth closely paralleled that of the same strains containing a mutation in the *sdh* gene (Fig. 3A and B); thus, these results were consistent with an *sdh* mutation causing its effect through increased levels of succinate.

When the strains shown in Fig. 4A and B were subcultured into identical media, however, the result was distinct from that seen with strains containing the *sdh* mutation. Although exogenous succinate did not allow thiamine-independent growth of DM587 (*purF apbA*) upon reinoculation, it did allow continuous growth of DM1936 (*purF*) (Fig. 4C and D and data not shown). This result suggested that if sufficient succinate was supplied, a *purF* mutant synthesized enough thiamine via the APB pathway to allow growth in glucose adenine medium. By this scenario, an Sdh block would not result in continued thi-

TABLE 4. Succinate allows increased thiamine-independent growth of *thi* mutants[a]

Strain (genotype)	Medium	A_{650} after 24 h of growth with thiamine supplement at final concn of:					
		1 μM	100 nM	10 nM	5 nM	1 nM	0
DM95 (*thiC885*::MudJ)	Minimal glucose	1.01	1.02	0.808	0.580	0.194	0.047
	Minimal glucose succinate	1.10	1.05	0.885	0.808	0.408	0.106
	Minimal glucose pantothenate	1.00	1.06	0.810	0.573	0.189	0.053
DM269 [*thiI887*::Tn*10*d(Tc)]	Minimal glucose	1.03	0.990	0.764	0.572	0.276	0.184
	Minimal glucose + succinate	1.10	1.02	0.875	0.845	0.550	0.426
	Minimal glucose + pantothenate	1.01	0.995	0.750	0.580	0.264	0.194

[a] A 0.15-ml volume of an overnight culture resuspended in saline was used to inoculate 5 ml of the indicated medium. Strains were grown at 37°C in a rotary shaker at 250 rpm. Data are representative of three independent experiments.

amine-independent growth because succinate accumulation was not sufficient.

Exogenous succinate increases thiamine-independent growth of *thi* mutants. To directly test whether succinate and/or pantothenate was affecting thiamine metabolism by a reduction in the cellular thiamine requirement, we tested the effect of these compounds on the growth of two defined thiamine auxotrophs that were defective in distinct areas of thiamine synthesis. DM269 contains a mutation in *thiI* (33) and specifically requires the thiazole moiety of thiamine, and DM95 contains a polar mutation in *thiC* and will grow only in the presence of thiamine or TPP (34). The data shown in Table 4 indicate that the growth of both of these thiamine auxotrophs was limited by available TPP when exogenous thiamine concentrations were below 10 nM. When succinate was added to the medium, both DM269 and DM95 grew to higher cell densities under thiamine-limiting conditions. In contrast, exogenous pantothenate did not significantly affect the growth of either mutant. These results suggest that (i) the effect of succinate on the growth of *purF* derivatives was due to a general effect on the cellular thiamine requirement and was not specifically related to HMP synthesis and (ii) pantothenate did not affect the cellular thiamine requirement and thus had an effect on thiamine metabolism separate from that of succinate.

A block in SucCD eliminates the effect of succinate. The results described above were consistent with an *sdh* mutation causing its phenotype by reducing the cellular thiamine requirement through an accumulation of succinate. However, the mechanism by which succinate accumulation had this effect was unclear. One possibility was that succinate accumulation resulted in succinyl-CoA accumulation by the action of succinyl-CoA synthetase (SucCD), a change that might be expected to reduce the need for α-ketoglutarate dehydrogenase, a TPP-dependent enzyme. Since strains defective in *sucCD* are unable to convert succinate to succinyl-CoA (Fig. 1B), we constructed a *purF sucCD* double mutant, DM2398, to determine whether the effect of succinate was due to its conversion to succinyl-CoA. In contrast to the *purF* mutant (DM1936), the *purF sucCD* mutant was unable to grow on glucose adenine medium supplemented with succinate (growth rate [μ] = 0.42 and 0.03, respectively). Both strains grew in the presence of thiamine (μ = 0.56 and 0.55, respectively). The fact that the *sucCD* mutation prevented the effect of succinate on thiamine-independent growth suggested that the effect of succinate was dependent on its conversion to succinyl-CoA.

Paradoxically, *purF sucCD* mutants were unable to grow on glucose adenine medium (μ = 0.06) in the absence of thiamine. This result was unexpected since a mutant blocked in SucCD would be expected to accumulate succinyl-CoA, possibly to a higher level than that of *sdh* mutants (see discussion).

Compounds that spare succinyl-CoA decrease the cellular thiamine requirement. To further address a model designating succinyl-CoA as the important metabolite, we tested whether compounds that would be expected to spare succinyl-CoA had the predicted effect on the thiamine requirement. The primary role of succinyl-CoA in *S. typhimurium* is to serve as a transient intermediate in lysine, methionine, and DAP biosynthesis (16, 25). Exogenous addition of these compounds would be expected to spare succinyl-CoA pools by eliminating the cellular requirement for succinyl-CoA. The effect of exogenous lysine, methionine, and DAP on the thiamine-independent growth of several thiamine auxotrophs is shown in Table 5. Three points can be made from these data. (i) Although some effects on thiamine-independent growth were observed when these compounds were added separately, the most dramatic changes in growth were seen when the three succinyl-CoA-dependent compounds were added together. (ii) As with previous experiments, the thiamine-independent growth observed was more pronounced in DM1936 (*purF*) than in DM587 (*purF apbA*). (iii) Unlike the succinyl-CoA-derived compounds that extended thiamine-independent growth in all of the thiamine-requiring strains, exogenous pantothenate allowed thiamine-independent growth only in *purF* and *purF apbA* mutants.

In a modification of the experiment described above, we

TABLE 5. Compounds that spare succinyl-CoA increase growth of thiamine auxotrophs under thiamine-limiting conditions[a]

Strain	Genotype	A_{650} after 24 h of growth in:						
		Glucose Ade	Glucose Ade Lys	Glucose Ade Met	Glucose Ade DAP	Glucose Ade Lys Met DAP	Glucose Ade Pan	Glucose Ade Thi
DM1936	*purF2085*	0.318	0.460	0.692	0.402	0.885	0.790	0.925
DM587	*purF2085 apbA7*::Tn*10*d(Tc)	0.057	0.073	0.050	0.077	0.584	0.760	0.965
DM95	*thiC885*::MudJ	0.020	0.035	0.035	0.050	0.266	0.040	0.865
DM269	*thiI887*::Tn*10*d(Tc)	0.141	0.189	0.150	0.151	0.383	0.102	0.955

[a] A 0.15-ml volume of an overnight culture resuspended in saline was used to inoculate 5 ml of the indicated medium. Strains were grown at 37°C in a rotary shaker at 250 rpm. Data shown are representative of three independent experiments. Abbreviations: Ade, adenine; Met, methionine; Lys, lysine; Pan, pantothenate; Thi, thiamine.

found that addition of succinyl-CoA-derived compounds also stimulated the growth of *thi* auxotrophs after their growth had ceased because of thiamine starvation. When DM95 and DM269 were grown for 24 h in limiting concentrations (1 nM) of thiamine, their growth ceased at optical densities of 0.175 and 0.245, respectively. The cell densities increased to 0.360 and 0.440, respectively, in another 24 h after addition of lysine, methionine, and DAP, while those of the control culture did not change significantly. This result suggested that TPP-starved cells ceased growth because of the lack of succinyl-CoA-derived metabolites.

Succinyl-CoA is not required for PurF-independent thiamine synthesis. Although the results presented above strongly suggested that the accumulation of succinyl-CoA affected growth of thiamine-requiring mutants by altering the cell's thiamine requirement, the possibility of succinyl-CoA being required for PurF-independent thiamine synthesis had not been ruled out. To test this idea, we constructed a strain unable to synthesize succinyl-CoA by any known route. This triple mutant (DM2408) was defective in the two enzymes able to generate succinyl-CoA, α-ketoglutarate dehydrogenase (SucAB) and succinyl-CoA synthetase (SucCD), in addition to PurF (Fig. 1B). As expected, this triple mutant required adenine, lysine, methionine, and DAP for growth. Significantly, this strain did not require thiamine, despite repeated reinoculations. This result confirmed that succinyl-CoA was not required for PurF-independent thiamine synthesis.

DISCUSSION

This work was initiated to identify the role of pantothenate in thiamine synthesis in *S. typhimurium*. We isolated derivatives of a *purF* mutant that grew in the absence of pantothenate or thiamine and found that they were defective in the reaction catalyzed by the Sdh enzyme. Further characterization of these mutants led us to conclude that the phenotype of *purF sdh* mutants was not due to pantothenate-independent thiamine synthesis but rather to a decrease in the cellular thiamine requirement. This conclusion was based primarily on two results: (i) growth of strains containing *sdh* mutations did not continue after subculturing into medium lacking pantothenate or thiamine, and (ii) conditions that imitated the effect of an *sdh* mutation, i.e., exogenous succinate, or lysine, methionine, and DAP, appeared to have a general effect on the thiamine requirement as evidenced by their ability to increase the growth of various *thi* mutants under thiamine-limiting conditions.

Based on results described herein, we propose a model to explain how an *sdh* mutation reduces the amount of thiamine required for growth. The model contains the following key points. (i) The α-ketoglutarate dehydrogenase enzyme is required for synthesis of biosynthetic precursors under aerobic conditions on glucose and sequesters a significant portion of cellular TPP. (ii) Conditions that either increase TPP-independent synthesis of the product of this reaction, succinyl-CoA, or eliminate the need for succinyl-CoA as an essential metabolite decrease the TPP requirement of the cell. (iii) An *sdh* mutation acts by preventing conversion of the succinate–succinyl-CoA pool into other cellular metabolites, i.e., subsequent TCA cycle intermediates. The Sdh block thus restricts use of succinyl-CoA to biosynthetic purposes, decreasing the need for TPP-dependent synthesis of succinyl-CoA.

One result from this study appeared inconsistent with the model described above. Although a lesion in succinyl-CoA synthetase (SucCD) would be expected to prevent conversion of the succinyl-CoA pool to other metabolites in a way similar to that of an Sdh block, a *sucCD* mutation did not result in a similar stimulation of the growth of a *purF* mutant. In fact, the thiamine-independent growth of a *purF sdh sucCD* mutant was identical to that of a *purF* mutant (data not shown), suggesting that a functional SucCD enzyme is required for the effect of an Sdh block. An attractive explanation for this result is that there is a source of succinate in the cell other than the TCA cycle, and this succinate contributes to the succinyl-CoA pool. Evidence for an alternative source of succinate has been reported in *Escherichia coli* (4, 5); our results are consistent with this conclusion.

The work described herein provides an explanation for several previous reports in the literature that cite positive effects of succinate or of lysine and methionine on the growth of thiamine auxotrophs (4, 23, 24). Addition of succinyl-CoA-derived compounds stimulated the growth of *thi* auxotrophs after their growth had ceased because of thiamine starvation. From these results, we concluded that cells starved for TPP stop growing because they lack compounds whose synthesis requires succinyl-CoA (e.g., lysine, methionine, and DAP).

Our results allowed us to conclude that although *sdh* mutations and pantothenate both stimulate thiamine-independent growth of a *purF* mutant, their effects on thiamine metabolism are distinct. We present substantial evidence indicating that an Sdh block decreases the cellular thiamine requirement, but our data do not support a similar role for pantothenate. Exogenous pantothenate allowed continuous thiamine-independent growth of *purF* and *purF apbA* mutants yet had no effect on defined *thi* mutants. These results are consistent with pantothenate stimulating thiamine synthesis in the absence of the PurF enzyme, although the mechanism of this stimulation remains unclear.

This study has increased our understanding of the requirement for thiamine in general metabolism. We have presented data showing that the need for α-ketoglutarate dehydrogenase function influences the cellular TPP requirement, even under conditions where this enzyme is strongly repressed (aerobic growth on glucose) (21, 30). In addition, we have shown that a block in another step of the TCA cycle, that catalyzed by succinate dehydrogenase, decreases the need for α-ketoglutarate dehydrogenase function. In addition, our data indicated TPP-starved cells cease growth because they lack essential succinyl-CoA-derived compounds. Thus, while the specific role of pantothenate in thiamine synthesis remains unclear, this work has resulted in an increased understanding of key aspects of thiamine metabolism in *S. typhimurium.*

ACKNOWLEDGMENTS

This work was supported by NIH grant GM 47296 to D.M.D. J.L.E. was supported by an NSF predoctoral fellowship and the Pfizer Fellowship in Microbial Physiology.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Berkowitz, D., J. M. Hushon, H. J. Whitfield, J. Roth, and B. N. Ames.** 1968. Procedure for identifying nonsense mutations. J. Bacteriol. **96:**215–220.
3. **Castilho, B. A., P. Olfson, and M. J. Casadaban.** 1984. Plasmid insertion mutagenesis and *lac* gene fusion with mini Mu bacteriophage transposons. J. Bacteriol. **158:**488–495.
4. **Creaghan, I. T., and J. R. Guest.** 1978. Succinate dehydrogenase-dependent nutritional requirement for succinate in mutants of *Escherichia coli* K-12. J. Gen. Microbiol. **107:**1–13.
5. **Creaghan, I. T., and J. R. Guest.** 1977. Suppression of the succinate requirement of lipoamide dehydrogenase mutants of *Escherichia coli* by mutations affecting succinate dehydrogenase activity. J. Gen. Microbiol. **102:**183–194.
6. **Cronan, J. E. J., and D. LaPorte.** 1996. Tricarboxylic acid cycle and gloxylate bypass, p. 206–216. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, Jr., B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter,

and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed., vol. 1. ASM Press, Washington, D.C.
7. **David, S., B. Estramareix, J. C. Fischer, and M. Therisod.** 1982. The biosynthesis of thiamine. Synthesis of [1,1,1,5-$^{2}H_{4}$]-1-deoxy-D-*threo*-2-pentulose and incorporation of this sugar in biosynthesis of thiazole by *Escherichia coli* cells. J. Chem. Soc. Perkin Trans. **1:**2131–2137.
8. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **DeMoll, E., and W. Shive.** 1985. Determination of the metabolic origin of the sulfur atom in thiamine of *Escherichia coli* by mass spectrometry. Biochem. Biophys. Res. Commun. **132:**217–222.
10. **Downs, D. M.** 1992. Evidence for a new, oxygen-regulated biosynthetic pathway for the pyrimidine moiety of thiamine in *Salmonella typhimurium*. J. Bacteriol. **174:**1515–1521.
11. **Downs, D. M., and L. Petersen.** 1994. *apbA*, a new genetic locus involved in thiamine biosynthesis in *Salmonella typhimurium*. J. Bacteriol. **176:**4858–4864.
12. **Downs, D. M., and J. R. Roth.** 1991. Synthesis of thiamine in *Salmonella typhimurium* independent of the *purF* function. J. Bacteriol. **173:**6597–6604.
13. **Enos-Berlage, J. L., and D. M. Downs.** 1996. Involvement of the oxidative pentose phosphate pathway in thiamine biosynthesis in *Salmonella typhimurium*. J. Bacteriol. **178:**1476–1479.
14. **Estramareix, B., and S. David.** 1990. Conversion of 5-aminoimidazole ribotide to the pyrimidine of thiamin in enterobacteria: study of the pathway with specificity labeled samples of riboside. Biochim. Biophys. Acta **1035:** 154–160.
15. **Estramareix, B., and M. Therisod.** 1972. Tyrosine as a factor in biosynthesis of the thiazole moiety of thiamine in *Escherichia coli*. Biochim. Biophys. Acta **273:**275–282.
16. **Greene, R. C.** 1996. Biosynthesis of methionine, p. 542–560. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, Jr., B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed., vol. 1. ASM Press, Washington, D.C.
17. **Guest, J. R.** 1981. Partial replacement of succinate dehydrogenase by phage and plasmid-specified fumarate reductase in *Escherichia coli*. J. Gen. Microbiol. **122:**171–179.
18. **Hong, J. S., and B. N. Ames.** 1971. Localized mutagenesis of any specific small region of the bacterial chromosome. Proc. Natl. Acad. Sci. USA **68:** 3158–3162.
19. **Jackowski, S.** 1996. Biosynthesis of pantothenic acid and coenzyme A, p. 687–694. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, Jr., B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed., vol. 1. ASM Press, Washington, D.C.
20. **Langley, D., and J. R. Guest.** 1974. Biochemical and genetic characteristics of deletion and other mutant strains of *Salmonella typhimurium* LT2 lacking α-keto acid dehydrogenase complex activities. J. Gen. Microbiol. **82:**319–335.
21. **Langley, D., and J. R. Guest.** 1978. Biochemical genetics of the α-keto acid dehydrogenase complexes of *Escherichia coli* K12: genetic characterization and regulatory properties of deletion mutants. J. Gen. Microbiol. **106:**103–117.
22. **Liu, S., and K. E. Sanderson.** 1992. A physical map of the *Salmonella typhimurium* LT2 genome made by using *Xba*I analysis. J. Bacteriol. **174:** 1662–1672.
23. **Nakayama, H.** 1971. Succinate requirement and thiamine deficiency of a thiamine-less mutant strain of *Escherichia coli* (II). The relationship of succinate to thiamine formation. Vitamins **43:**17–22.
24. **Nakayama, H., and K. Kuba.** 1970. Succinate requirement and thiamine deficiency of a thiamine-less mutant strain of *Escherichia coli* (I). Thiamine deficiency of a mutant cell grown anaerobically on succinate. Vitamins **41:** 165–172.
25. **Patte, J.-C.** 1996. Biosynthesis of threonine and lysine, p. 528–541. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, Jr., B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed., vol. 1. ASM Press, Washington, D.C.
26. **Petersen, L., and D. M. Downs.** 1996. Mutations in *apbC* (*mrp*) prevent function of the alternative pyrimidine biosynthetic pathway in *Salmonella typhimurium*. J. Bacteriol. **178:**5676–5682.
27. **Petersen, L. A., J. L. Enos-Berlage, and D. M. Downs.** 1996. Genetic analysis of metabolic crosstalk and its impact on thiamine synthesis in *Salmonella typhimurium*. Genetics **143:**37–44.
28. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
29. **Schmieger, H.** 1972. Phage P22 mutants with increased or decreased transduction abilities. Mol. Gen. Genet. **119:**75–88.
30. **Smith, M. W., and F. C. Neidhardt.** 1983. 2-Oxoacid dehydrogenase complexes of *Escherichia coli*: cellular amounts and patterns of synthesis. J. Bacteriol. **156:**81–88.
31. **Spencer, M. E., and J. R. Guest.** 1973. Isolation and properties of fumarate reductase mutants of *Escherichia coli*. J. Bacteriol. **114:**563–570.
32. **Way, J. C., M. A. Davis, D. Morisato, D. E. Roberts, and N. Kleckner.** 1984. New Tn*10* derivatives for transposon mutagenesis and for construction of *lacZ* operon fusions by transposition. Gene **32:**369–379.
33. **Webb, E., K. Claas, and D. Downs.** Characterization of *thiI*, a new gene involved in thiazole biosynthesis in *Salmonella typhimurium*. J. Bacteriol., in press.
34. **Webb, E., F. Febres, and D. M. Downs.** 1996. Thiamine pyrophosphate (TPP) negatively regulates transcription of some *thi* genes of *Salmonella typhimurium*. J. Bacteriol. **178:**2533–2538.
35. **Wilde, R. J., and J. R. Guest.** 1986. Transcript analysis of the citrate synthase and succinate dehydrogenase genes of *Escherichia coli* K12. J. Gen. Microbiol. **132:**3239–3251.
36. **Zilles, J. L., and D. M. Downs.** 1996. A novel involvement of the PurG and PurI proteins in thiamine synthesis via the alternative pyrimidine biosynthetic (APB) pathway in *Salmonella typhimurium*. Genetics **144:**883–892.