JOURNAL OF BACTERIOLOGY, Aug. 1997, p. 4894–4900 Vol. 179, No. 15
0021-9193/97/$04.00+0
Copyright © 1997, American Society for Microbiology

Identification and Characterization of an Operon in *Salmonella typhimurium* Involved in Thiamine Biosynthesis

LESLIE A. PETERSEN AND DIANA M. DOWNS*

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

Received 6 March 1997/Accepted 30 May 1997

Thiamine pyrophosphate (TPP) is synthesized de novo in *Salmonella typhimurium* and is a required cofactor for many enzymes in the cell. Five kinase activities have been implicated in TPP synthesis, which involves joining a 4-methyl-5-(β-hydroxyethyl)thiazole (THZ) moiety and a 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP) moiety. We report here identification of a 2-gene operon involved in thiamine biosynthesis and present evidence that the genes in this operon, *thiMD*, encode two previously identified kinases, THZ kinase and HMP phosphate (HMP-P) kinase, respectively. We further show that this operon belongs to the growing class of genes involved in TPP synthesis that are transcriptionally regulated by TPP. Our data are consistent with ThiM being a salvage enzyme and ThiD being a biosynthetic enzyme involved in TPP synthesis, as previously suggested.

Thiamine pyrophosphate (TPP) is an essential cofactor for many enzymes in the cell, including pyruvate dehydrogenase, transketolase, and α-ketoglutarate dehydrogenase. Despite the critical role of this vitamin in metabolism, its biosynthetic pathway is poorly understood. Our current understanding of thiamine synthesis in *Salmonella typhimurium* is summarized in Fig. 1. TPP is generated by the phosphorylation of thiamine monophosphate (TMP) by TMP kinase (ThiL) (40). TMP, in turn, is formed by the action of thiamine phosphate pyrophosphorylase (ThiE), which couples a 4-methyl-5-(β-hydroxyethyl) thiazole (THZ) monophosphate moiety (THZ-P) and a 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP) pyrophosphate moiety (HMP-PP) (2). The pathways for the biosynthesis of the HMP and THZ moieties are separate, and their characterization is in the early stages. Labeling studies have determined that cysteine, tyrosine, and deoxyxylulose contribute to the THZ moiety (9, 11, 15).

In *Escherichia coli* and *S. typhimurium*, aminoimidazole ribotide (AIR) is a common precursor for both HMP and purine biosynthesis (26, 28). In at least *S. typhimurium*, HMP can be synthesized independently of de novo purine synthesis, through a proposed AIR intermediate (12, 13, 31, 32). Evidence has been presented that there are two routes for the conversion of AIR to HMP, but the contribution of each to thiamine synthesis is unclear (31). In contrast, two pathways for the synthesis of the HMP moiety have been identified in yeast, yet neither of these pathways uses AIR as an intermediate (3).

Genes involved in the synthesis of thiamine map to several locations. An operon containing 5 genes designated *thiCEFGH* that is transcriptionally regulated by TPP has been described (36, 41). Of the gene products from this operon, ThiE has been shown in vitro to catalyze the condensation of HMP-PP and THZ-P to yield TMP (2) and ThiG catalyzes the epimerization of deoxyxylulose-5-phosphate, a proposed intermediate in THZ synthesis (4). Lesions in either *thiF* or *thiH* cause a growth requirement for THZ, and mutants defective in *thiC* require HMP (3, 36, 41). An additional single-gene locus, *thiI*, required for THZ synthesis has been described at 10 centrosomes (Cs) on the *S. typhimurium* chromosome (39).

In addition to the above gene products, five kinases that are involved in the formation of TPP have been identified. The *thiL* gene (10 Cs) encodes TMP kinase (17, 40), and *thiK* (25 Cs), *thiM*, *thiJ* (*N*), and *thiD* (46 Cs) have been proposed to encode thiamine kinase, THZ kinase, HMP kinase, and HMP-P kinase, respectively (Fig. 1) (5, 16, 17, 19, 22, 24). Previously, *thiN* was called *thiJ* (23).

The role of the above kinases in thiamine biosynthesis is complex and not completely understood. It is not clear whether THZ and HMP or the corresponding phosphate esters are the products of the de novo synthetic pathways. It has been suggested that ThiK, ThiM, and ThiJ are salvage enzymes, and it has been predicted that HMP-P and THZ-P are the true products of de novo synthesis (3, 17, 22, 24, 25). In support of their designation as salvage enzymes, both *thiK* and *thiM* point mutants are reported to be prototrophic in *E. coli* (24). Additionally, in vitro activity of ThiG required a phosphorylated substrate, suggesting that phosphorylation occurred in the pathway prior to the formation of THZ (4). In parallel, one of the proposed chemical mechanisms for the synthesis of HMP-P utilizes a phosphorylated intermediate, consistent with ThiJ performing a salvage function (3).

In contrast to the kinases described above, both *thiD* and *thiL* appear to be essential for the synthesis of TPP. Mutations in these genes resulted in a requirement for thiamine or TPP, respectively (16, 40).

This report describes the identification and characterization of a 2-gene operon at 46 Cs on the *S. typhimurium* chromosome that is involved in thiamine biosynthesis. Data presented showed that *thiM* is in an operon with *thiD* and further that this operon is transcriptionally regulated by TPP. Our data are consistent with previous reports that ThiM (thiazole kinase) is a salvage enzyme and ThiD (HMP-P kinase) has a biosynthetic role in TPP synthesis. Based on phenotypic analysis, we suggest that this operon has an internal promoter to ensure transcription of *thiD*.

MATERIALS AND METHODS

Bacterial strains. All strains in this study are derived from *S. typhimurium* LT2 unless otherwise noted and are described in Table 1. Tn*10d*(Tc) refers to the transposition-defective mini-Tn*10* described by Way et al. (38). MudJ is used throughout this report to refer to the MudI1734 transposon, which has been described previously (6).

* Corresponding author. Mailing address: Department of Bacteriology, 1550 Linden Dr., Madison, WI 53706. Phone: (608) 265-4630. Fax: (608) 262-9865. E-mail: downs@vms2.macc.wisc.edu.

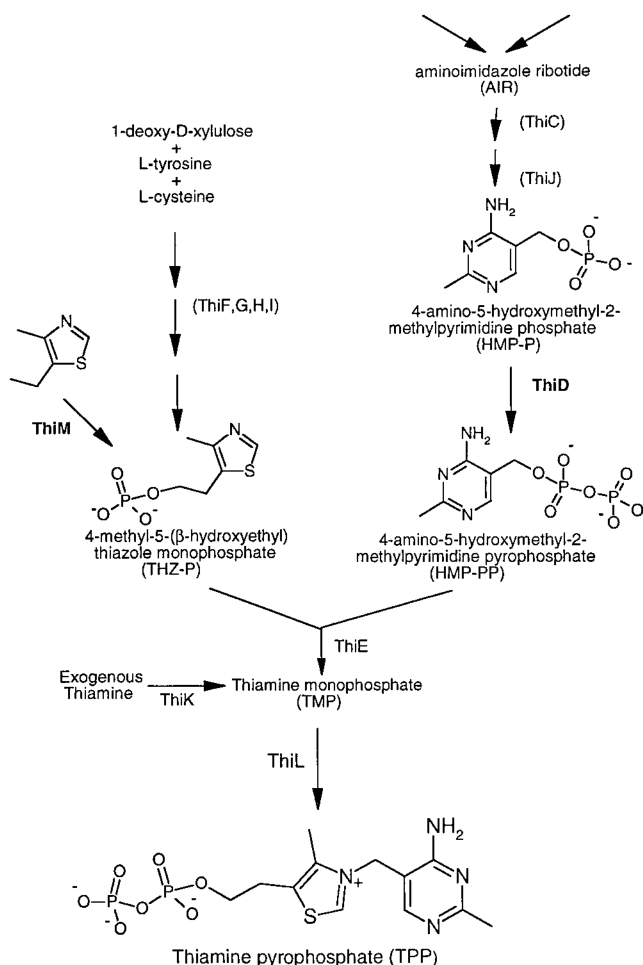

FIG. 1. Biosynthetic route for the synthesis of TPP. A schematic shows the biosynthetic routes of HMP-PP and THZ-P. The biosynthetic steps between 1-deoxy-D-xylulose and THZ and between AIR and HMP are unknown. The *thiE* gene product couples THZ-P and HMP-PP to form TMP (2). TMP is phosphorylated by ThiL to form TPP.

Culture media and chemicals. No-carbon E medium supplemented with $MgSO_4$ (1 mM) (10, 37) and D-gluconic acid (11 mM for aerobic growth and 22 mM for anaerobic growth) was used as a carbon source. Difco nutrient broth (8 g/liter) with NaCl (5 g/liter) was used as nutrient broth (NB) rich medium. Luria broth (LB) consisted of Difco Bacto Tryptone (10 g/liter) and yeast extract (5 g/liter) with NaCl (5 g/liter). For solid medium Difco Bitek agar was added (15 g/liter). Adenine was added to a final concentration of 0.4 mM; thiamine, TPP, THZ, and HMP were added to a final concentration of 100 nM. The final concentrations of the antibiotics in rich medium were as follows (in micrograms per milliliter): ampicillin, 30; chloramphenicol, 20; kanamycin, 50; and tetracycline, 20. In minimal medium the final concentrations were 15, 4, 125, and 10 μg/ml, respectively. All chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.), with the exception of isopropyl-β-D-thiogalactopyranoside (IPTG), which was purchased from Fisher Scientific (Chicago, Ill.). HMP was kindly provided by Tadhg Begley, Cornell University.

Transduction methods. Transductions were performed with the high-frequency, generalized transducing mutant HT105/1 *int201* of bacteriophage P22 (34) as described previously (13). Transductants were purified and identified as phage free by streaking and cross-streaking on green plates (7).

Mutant isolation. Mutants with a Thi^- phenotype were isolated by insertion mutagenesis as described previously by using either Tn*10d*(Tc) or MudJ (31, 41). Once the *thiMD* locus was defined, additional insertions were isolated with two localized techniques. Transposition by *cis*-complementation was used to generate a pool of >70,000 cells containing independent MudJ insertions. This pool of cells was generated in LB to ensure representation of mutants impaired for growth on NB (13, 20). A P22 lysate grown on this pool of cells was used to transduce SA2026 (*metG419*) to kanamycin resistance (Km^r) on LB medium. Since the *metG* locus is >50% linked to the *thi* locus of interest, the Km^r transductants were screened for growth without methionine in the presence of thiamine. The Km^r Met^+ transductants were further screened for Met^+ Thi^- isolates. In addition, Tn*10d*(Tc) insertions in the *thiMD* locus were generated by using a phage lysate on a pool of >100,000 cells containing Tn*10d*(Tc) elements (38) to transduce DM176 (*thiM938*::MudJ) to tetracycline resistance (Tc^r) on LB medium. Tc^r Km^s transductants were screened for those with a Thi^- phenotype. Putative mutants in each case were reconstructed by transducing the relevant insertion into a fresh genetic background to confirm the original phenotype.

Phenotypic characterization. Mutant phenotypes were assessed by liquid growth curves and solid media tests as described previously (31). Specific growth rate was determined as $m = \ln(X/X_0)/T$, where X is A_{650} during the linear portion of the growth curve and T is time.

Cloning *thiMD*. A clone complementing *thiD* was obtained from an *S. typhimurium* chromosomal plasmid library derived from pBR328 (from C. Miller, University of Illinois). A P22 lysate grown on cells containing the plasmid library was used to transduce strain DM456 (*thiD906*::MudJ) to Amp^r on rich medium. Putative complementing clones were identified by the ability of the resulting strain to grow on minimal medium. Plasmids were purified with a QIAPrep Spin Plasmid kit (Qiagen, Chatsworth, Calif.) and reintroduced into mutant strains via electroporation (Bio-Rad Laboratories, Richmond, Calif.). A clone with a ca. 4.5-kbp insert that complemented the Thi^- phenotype of DM456 (*thiD906*::MudJ) was isolated and designated pThiM. A 2.5-kbp *Hin*dIII fragment from pThiM was purified with the Qiagen band extraction kit and ligated into the multiple cloning site (MCS) of pSU19. The resulting clone complemented the Thi^- phenotype of both DM456 (*thiD906*::MudJ) and DM176 (*thiM938*::MudJ) and was designated pThiM1 (Fig. 2).

DNA sequencing. (i) Plasmid. The insert of pThiM1 was sequenced by the dideoxynucleotide chain termination method (33) with modified T7 DNA polymerase (United States Biochemicals, Cleveland, Ohio) and ^{35}S-dATP with a specific activity of 1,000 to 1,500 Ci/mmol (Dupont, Beverly, Mass.). Primers were designed as sequence was determined and then were generated by Genosys (The Woodlands, Tex.). Primers were designed as follows: **pBR** (5′ GCGATC ATGGCGACCACACCCGTC 3′), **F1** (5′ AGGCAGTATACACCACCACGC 3′), **F2** (5′ GACGCGGGTAAAGAGCCAGT 3′), **F3** (5′ CGTAATGTTTCTA TCGC 3′), **F4** (5′ CAGTGATGACCGAACAGC 3′), **R1** (5′ CATATCGCCA GCAAGTTGCC 3′), **R2** (5′ ATGCCAGCGTAGGGAAGTCT 3′), **R3** (5′ CTT ATGATGCCAAAACG 3′), **R4** (5′ TTACCGGAGAGATAGATT 3′), and **R5** (5′ TCCCGGCTTTTCTTGATG 3′). Sequence was confirmed at the University of Wisconsin, Biotechnology Center, Nucleic Acid and Protein Facility (Madison, Wis.).

(ii) Amplified products. Amplified PCR products were sequenced by using the Sequitherm cycle sequencing kit from Epicentre Technologies Corporation (Madison, Wis.) and ^{35}S-dATP with a specific activity of 1,000 to 1,500 Ci/mmol.

Computer analysis. BLAST (Basic Local Alignment Search Tool) was used to search for homologous sequences of DNA in the nonredundant GenBank and

TABLE 1. Strains used in this study

Strain	Genotype
LT2	Wild type
DM95	*thiE918*::MudJ[a]
DM456	*thiD906*::MudJ
DM912	*purF2085 thiM938*::MudJ
DM997	*thiD906*::MudJ/pThiM1
DM930	*purF2085 apbC55*::Tn*10d*[b]
DM1142	*purF2085 thiD906*::MudJ
DM1390	*thiM937*::Tn*10d*
DM1955	*thiM938*::MudJ/pThiM1
DM2835	*thiD906*::MudJ/pThiM4
DM2836	*thiM938*::MudJ/pThiM4
DM3062	LT2/pThiM1
DM3168	*thiM976*::MudJ
DM3169	*thiM937*::MudJ
DM3170	*thiM976*::MudJ/pThiM1
DM3171	*thiM977*::MudJ/pThiM1
DM3174	*thiM937*::Tn*10d*(Tc) *thiM977*::MudJ
DM3204	*thiD906*::MudJ *thiM937*::Tn*10d*(Tc)
DM3205	*thiD906*::MudJ
DM3208	*thiD906*::MudJ *thiM937*::Tn*10d*(Tc)
DM3209	*thiD906*::MudJ/pThiM1
DM3212	*thiM979*::MudJ
DM3213	*thiM980*::MudJ
BL21/IDE3	F^- *ompT hsdS*$_B$ (r_B^- m_B^-) *gal dcm*
DM2717	BL21/IDE3/pThiM3 (*E. coli*)
DM2718	BL21/IDE3/pThiM3 (*E. coli*)

[a] MudJ is used throughout the text to refer to the MudI1734 transposon (6).
[b] Tn*10d* refers to the transposition-defective mini-Tn*10* (Tn*10*Δ16Δ17) (38).

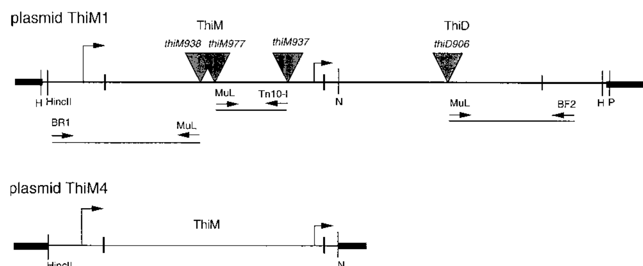

FIG. 2. Plasmid construction and mapping of *thiMD* insertions. Plasmid pThiM4 was constructed by digesting pThiM1 with *Nae*I and *Hin*CII and ligating that fragment into *Sma*I-digested pSU19. Thick lines represent plasmid sequences, and narrow lines represent *S. typhimurium* insert DNA. The locations of four insertions are indicated. PCR amplification products which were sequenced are indicated with their respective primers. H, *Hin*dIII; N, *Nae*I; P, *Pst*I; S, *Sma*I.

Swissprot databases (1). The DNASTAR alignment program (Madison, Wis.) was used to line up regions of homology.

Overexpression of ThiM and ThiD. The *Hinc*II/*Pst*I fragment from pThiM1 (Fig. 2) was cloned into the *Sma*I-*Pst*I sites of T7 overexpression vectors pT7-5 and pT7-6, which differ in the orientation of the MCS with respect to the T7 promoter (35). The resulting plasmids (pThiM3 and pThiM2) were electroporated into *E. coli* BL21/IDE3 [F$^-$ *ompT hsdS*$_B$ ($r_B{}^-$ $m_B{}^-$) *gal dcm* (DE3)], which carries the gene for T7 polymerase on a lambda lysogen under the control of an IPTG-inducible promoter. Specific labeling was done in vivo according to the protocol for expression with the T7 RNA polymerase/promoter system (35). The final IPTG concentration was 400 μM. Proteins expressed from pThiM3 were labeled with [^{35}S]methionine, analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with the Bio-Rad MiniProtean electrophoresis system, and visualized with a PhosphorImager.

Localization of Thi$^-$ insertions by PCR amplification. The locations of various insertions were determined by using a PCR protocol to amplify between known DNA sequences and the relevant insertion element, as described previously (8). Amplification between two primers was performed by using Vent (exo$^-$) polymerase (New England Biolabs) in a Thermolyne Temp-Tronic thermocycler. PCR conditions were as follows: denaturation at 95°C for 1 min, annealing at 59°C for 1 min, and extension at 72°C for 1.4 min. MuL (5′ ATCCCGAATAATCCAATGTCC 3′) hybridizes to the left end of the MudJ insertion, and Tn*10*-I (5′ GACAAGATGTGTATCCACCTTAAC 3′) hybridizes to the insertion sequences of Tn*10d*(Tc) (8).

Identification of HMP. HMP was purified by modifying a previously reported procedure (18). Briefly, 1 ml of an overnight culture of DM176 (*thiM938*::MudJ) was used to inoculate 2 liters of minimal gluconate medium and left stagnant to grow for 2 days at 37°C. The cells were pelleted by centrifugation, and the supernatant was autoclaved for 30 min. Six grams of activated charcoal was added, and the mixture was stirred for 1 h. After stirring, the mixture was filtered over Whatman no. 3 paper with a Büchner funnel and the filtrate was discarded. The charcoal was then washed with distilled water prior to two elutions with 250 ml of 95% ethanol-ammonium hydroxide (9:1). This eluant was concentrated to dryness, and the residue was dissolved in 20 ml of 0.1 M acetate buffer, pH 4.5. The 20-ml solution was applied to a column of Amberlite IRC-50 resin (1.0 by 15.0 cm; Aldrich Chemical, Milwaukee, Wis.) which had been equilibrated with 1 M acetate buffer, pH 4.5. The column was washed with 100 ml of distilled water and 100 ml of 0.01 N HCl. Elution of HMP was carried out with a linear gradient of HCl (0.01 to 0.03 N), collecting 5-ml fractions. A 5-μl aliquot from each fraction was tested for biological activity. Bioactivity was demonstrated by growth of DM930 *purF2085 apbC55*::Tn*10d*(Tc), an HMP-requiring strain.

Fractions from the Amberlite column that contained biological activity were combined, dried, and resuspended in distilled water. A 25-μl aliquot (pH 8.0) was applied to a Phenomenex (Torrance, Calif.) Magellan C_{18} (250 by 4.60 mm; inside diameter, 5 μm) column by injection into a Waters 600 high-pressure liquid chromatograph (HPLC). Elution was monitored at 270 nm by detection with a Waters 996 photodiode array detector. The active fraction was eluted from the column with a gradient of acetonitrile and water as follows: 100% water for 10 min, linear gradient from 0 to 15% acetonitrile in 10 min, and 15% acetonitrile for 10 min. Fractions of 2 ml were collected, dried, and resuspended in 100 μl of double-distilled water. Five microliters of each fraction was then spotted on DM930 [*purF2085 apbC55*::Tn*10d*(Tc)] to assess biological activity. Under these conditions, the biologically active fraction had a retention time of 24 min.

Spectral analysis was performed at the University of Wisconsin Protein and Mass Spectroscopy Facility and the Midwest Regional Nuclear Magnetic Resonance Facility at the University of Wisconsin—Madison.

β-Galactosidase assays. β-Galactosidase assays were performed by the method of Miller (21) as reported previously (14).

Nucleotide sequence accession number. The nucleotide sequence of *thiMD* has been submitted to GenBank as accession no. U87940.

RESULTS

Initial observations. During other work to identify genes involved in thiamine biosynthesis, Thi$^-$ mutants were isolated by insertion mutagenesis in different genetic backgrounds. One class of these mutants (3 of 53) was distinct in that the strains were impaired for growth on NB. Although NB is known to be low in thiamine (41), all other *thi* auxotrophs showed wild-type growth in this medium. This growth defect was relieved by the addition of thiamine or by growth on LB medium, consistent with an increased requirement for thiamine. This class of mutants was also distinct from the others in that thiamine, but neither the THZ nor the HMP moiety nor both, restored their growth on minimal medium. This nutritional requirement would be expected for mutants defective in thiamine phosphate pyrophosphorylase or any of the kinases involved in the de novo synthesis of THZ-P or HMP-PP. One such mutant (DM95) was found to be defective in *thiE* (encoding thiamine phosphate pyrophosphorylase) and was not further characterized here (41).

Mapping of *thiM* and *thiD* mutants. Two mutants, DM456 (*thiD906*::MudJ) and DM176 (*thiM938*::MudJ), were characterized, and both of the respective insertions were mapped to 46.6 Cs on the *S. typhimurium* chromosome based on transductional linkage to *apbC* (31).

Despite their similar map positions, the two mutants were phenotypically distinct. On solid NB medium strain DM176 (*thiM938*::MudJ) formed very small colonies, while strain DM456 (*thiD906*::MudJ) failed to form colonies. Similarly, when overnight growth in liquid NB was monitored, DM456 and DM176 reached A_{650}s of 0.212 and 0.965, respectively. After overnight incubation in minimal medium, a liquid culture of strain DM456 remained at an A_{650} of 0.011 whereas DM176 had reached an A_{650} of 0.341. These low yields represented low growth rates rather than an early entrance into stationary phase (data not shown). Exogenous thiamine restored wild-type growth of both mutants on NB and minimal medium. These results were consistent with the insertion in DM176 resulting in an incomplete thiamine auxotrophy. Three *thi* genes, *thiM*, *thiJ*, and *thiD*, had previously been mapped to this region of the chromosome in *E. coli* (16, 22–24), and phenotypic results suggested that we had identified mutations in more than one of these genes.

It was unclear why mutants that required the complete thiamine molecule should have impaired growth on NB. Wild-type growth of *thi* mutants defective in either HMP or THZ synthesis on NB medium ruled out the possibility that thiamine levels in NB were limiting for growth (41). We considered the possibility that accumulation of a phosphorylated intermediate in thiamine synthesis was toxic to the cell. In theory, this toxicity could be relieved by exogenous thiamine causing repression of endogenous thiamine synthesis (41). To address this possibility, we introduced a *purF* mutation into DM456. The *purF* mutation would block the major carbon flux to HMP synthesis and thus reduce, if not eliminate, accumulation of intermediates in the HMP branch. The *purF thiM* double mutant remained impaired for growth on NB, as did a *thiM* mutant derivative blocked in THZ synthesis. These results did not support toxic intermediate accumulation as an explanation for the growth defect of these mutants on NB medium, and they left us with no obvious explanation for this phenotype.

Sequencing of *thiM* and *thiD*. A clone (pThiM1) that complemented the Thi$^-$ phenotype of both DM456 (*thiD906*::MudJ) and DM176 (*thiM938*::MudJ) was constructed, and the 2.5-kbp insert was sequenced in its entirety on both strands.

Sequence analysis of the insert identified two consecutive

```
   1                                   CTCGGGGTGCCCTTCTTTGTGAAGGCTGAGAAATACCCGTA
  42 CCACCTGATCTGGATAATGCCAGCGTAGGGAAGTCTGACACTGCTCGTGTCCCTTCTTCACGCGGGCAGGAGCTGAACC
 121 ATG CAG CCT GAC CTG CAC TGC CGC ACG CTT GCG GCG CAT ACG TTA AAA CAC TTT CGC GCG
      M   Q   P   D   L   H   C   R   T   L   A   A   H   T   L   K   H   F   R   A
 181 CTC TCC CCG CTT ACG CAC TGT ATG ACG AAT GAC GTC GTA CAA ACG TTT ACC GCC AAT ACG
      L   S   P   L   T   H   C   M   T   N   D   V   V   Q   T   F   T   A   N   T
 241 TTG CTG GCG CTG GGC GCT TCA CCC GCC ATG GTG ATT GAT CCT GTC GAG GCC AGA CCG TTT
      L   L   A   L   G   A   S   P   A   M   V   I   D   P   V   E   A   R   P   F
 301 GCC GCC ATC GCC AAC GCC TTG CTG ATT AAT GTC GGA ACA TTA ACT GCC TCA CGC GCT GAC
      A   A   I   A   N   A   L   L   I   N   V   G   T   L   T   A   S   R   A   D
 361 GCG ATG CGT GCG GCG GTA GAA AGC GCT TAT GAT GCC AAA ACG CCG TGG ACG CTT GAT CTC
      A   M   R   A   A   V   E   S   A   Y   D   A   K   T   P   W   T   L   D   P
 421 GTC GCG GTG GGC GCG CTG GAA TTT CGT CGG CGA TTT TGC CTG GAT CTC TTG TCC CTG CGC
      V   A   V   G   A   L   E   F   R   R   R   F   C   L   D   L   L   S   L   R
 481 CCG GCG GCA ATA CGC GGC AAC GCC TCG GAA ATC CTG GCG TTA TCC GGC ATG GCG CTG GGC
      P   A   A   I   R   G   N   A   S   E   I   L   A   L   S   G   M   A   L   G
 541 GGA CGT GGC GTA GAT ACC ACC GAG GCG GCG TTG GCC GCA CTG CCT GCG GCG CAG GCG CTG
      G   R   G   V   D   T   T   E   A   A   L   A   A   L   P   A   A   Q   A   L
 601 GCG CGT CAG ATA GAC TGC ATC GTT GTG GTT ACC GGA GAG ATA GAT TAC GTC ACT AAT GGT
      A   R   Q   I   D   C   I   V   V   V   T   G   E   I   D   Y   V   T   N   G
 661 CAG CGC ACG CTG AGC ATT CCC GGC GGC GAT CCG TTA ATG ACT CGC ATT GTA GGC ACC GGC
      Q   R   T   L   S   I   P   G   G   D   P   L   M   T   R   I   V   G   T   G
 721 TGC GCG TTG TCG GCG GTC GTC GCC GCC AGT TGC GCG TTA CCG GGC GCC GCG CTG GAC AAT
      C   A   L   S   A   V   V   A   A   S   C   A   L   P   G   A   A   L   D   N
 781 GTC GCG TCG GCC TGC TGC TGG ATG AAA CTG GCT GGA CAG GCC GCG GCA GAG CGT AGC GAA
      V   A   S   A   C   C   W   M   K   L   A   G   Q   A   A   A   E   R   S   E
 841 GGA CCG GGT AGC TTC ATC CCG GCT TTT CTT GAT GCG CTC TAT CAT CTG GAT GTG GAG GCG
      G   P   G   S   F   I   P   A   F   L   D   A   L   Y   H   L   D   V   E   A
 901 GCC AAT GCA ACG AAT TAA C GCG CTG ACG ATT GCC GGC ACC GAT CCC AGC GGC GGG GCC GGT
      A   N   A   T   N   *
       thiD M   Q   R   I   N   A   L   T   I   A   G   T   D   P   S   G   G   A   G
 962 ATC CAG GCG GAT CTC AAG ACC TTT TCC GCG CTG GGC GCA TAT GGC TGT TCG GTC ATC ACT
      I   Q   A   D   L   K   T   F   S   A   L   G   A   Y   G   C   S   V   I   T
1022 GCG CTG GTG GCC GAA AAT ACC TGT GGC GTA CAG TCG GTG TAC CGG ATA GAG CCG GAC TTT
      A   L   V   A   E   N   T   C   G   V   Q   S   V   Y   R   I   E   P   D   F
1082 GTT GCC GCT CAA CTT GAT TCC GTG TTC AGC GAT GTG CGC ATT GAT ACC ACG AAA ATC GGG
      V   A   A   Q   L   D   S   V   F   S   D   V   R   I   D   T   T   K   I   G
1142 ATG CTG GCG GAA ACC GAT ATT GTC GAG GCC GTG GCG GAA CGG TTA CAG CGC CAT CAT GTA
      M   L   A   E   T   D   I   V   E   A   V   A   E   R   L   Q   R   H   H   V
1202 CGT AAT GTG GTG CTG GAT ACG GTG ATG CTG GCG AAA AGC GGC GAT CCG CTG CTC TCG CCC
      R   N   V   V   L   D   T   V   M   L   A   K   S   G   D   P   L   L   S   P
1262 TCT GCG ATA GAA ACA TTA CGC GTC CGG CTG TTG CCG CAG GTA TCG CTG ATT ACG CCT AAT
      S   A   I   E   T   L   R   V   R   L   L   P   Q   V   S   L   I   T   P   N
1322 TTG CCG GAA GCC GCA GCG TTG CTG GAT GCG CCC CAT GCG CGT ACG GAA CAG GAG ATG CTG
      L   P   E   A   A   A   L   L   D   A   P   H   A   R   T   E   Q   E   M   L
1382 GCG CAG GGG CGG GCG TTA CTG GCG ATG GGG TGT GAA GCG GTA TTG ATG AAA GGC GGG CAT
      A   Q   G   R   A   L   L   A   M   G   C   E   A   V   L   M   K   G   G   H
1442 TTA GAG GAT GCG CAA AGC CCG GAC TGG CTC TTT ACC CGC GAG GGC GAG CAG CGT TTT AGC
      L   E   D   A   Q   S   P   D   W   L   F   T   R   E   G   E   Q   R   F   S
1502 GCG CCG CGT GTG AAC ACC AAA AAT ACG CAT GGG ACG GGC TGT ACG CTG TCG GCG GCG CTG
      A   P   R   V   N   T   K   N   T   H   G   T   G   C   T   L   S   A   A   L
1562 GCG GCG TTA CGG CCC CGG CAT CGC AGT TGG GGA GAG ACG GTA AAC GAG GCG AAG GCA TGG
      A   A   L   R   P   R   H   R   S   W   G   E   T   V   N   E   A   K   A   W
1622 CTT TCG GCG GCG CTG GCG CAG GCG GAC ACG CTG GAA GTG GGG AAG GGC ATT GGT CCG GTA
      L   S   A   A   L   A   Q   A   D   T   L   E   V   G   K   G   I   G   P   V
1682 CAT CAT TTC CAC GCG TGG TGG TAG  1706
      H   H   F   H   A   W   W   *
```

FIG. 3. Nucleotide sequence of *thiMD*, determined by sequencing with the dideoxy method as described in Materials and Methods.

open reading frames (ORFs) of 795 and 798 bp and predicted that they encoded two proteins, of 27.4 and 28.5 kDa, respectively. These two ORFs were designated ThiM and ThiD, respectively, for reasons discussed below. The sequence of the ORFs is shown in Fig. 3.

As seen in Fig. 4, the ORF designated ThiM had significant homology to the C-terminal ends of both the *thi4* gene in *Schizosaccharomyces pombe* and the *thi6* gene in *Saccharomyces cerevisiae*. Both of these genes encode bifunctional enzymes with hydroxyethylthiazole kinase and thiamine phosphate pyrophosphorylase activity (30, 43). The C-terminal region of Thi6 from amino acid 370 to amino acid 453 has been shown to be responsible for the THZ kinase activity (30), and an amino acid change at residue 370 from glutamic acid to lysine resulted in decreased THZ kinase activity in the mutant protein. ThiM from *S. typhimurium* has the conserved glutamic acid residue and homology only to the C-terminal end of the Thi6 protein. Additionally, end-to-end homology is seen between ThiM and the *ywbJ* ORF from *Bacillus subtilis*. The product of this ORF has recently been shown to possess THZ kinase activity, and the ORF has been redesignated *thiK* (42). This sequence homology, in combination with the linkage and phenotypic data, led us to conclude that ORF1 was *thiM* and encoded hydroxyethylthiazole kinase. Based on the mutant phenotype and map location, we suggested that ORF2 encoded the hydroxymethylpyrimidine phosphate kinase and designated it *thiD*.

Interestingly, the stop codon of *thiM* fell 8 bp within the proposed coding region of *thiD*. Subsequent cloning experiments confirmed the assignment of the start codon for ThiD (data not shown). A similar overlap of consecutive genes is seen in the *thi* operon at 90 min between *thiF* and *thiG*, but no regulatory features have been attributed to this overlap (36).

Overexpression of ThiM and ThiD. In order to confirm that these two ORFs produced the predicted proteins, we cloned the *Hin*cII/*Pst*I fragment from pThiM1 into the T7 overexpression vectors pT7-6 and pT7-5, generating pThiM2 and pThiM3, respectively (35). These two clones were electroporated into BL21/ID3, an *E. coli* strain containing the gene for T7 RNA polymerase on a lambda lysogen. This resulted in strains DM2718 and DM2717, used for specific labeling experiments with [^{35}S] methionine as described in Materials and Methods. Proteins in crude cell extracts were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and labeled proteins were visualized with a PhosphorImager.

Sequence data predicted that ThiM was a protein of 265 amino acids with a molecular mass of 27.4 kDa and ThiD was a protein of 266 amino acids with a molecular mass of 28.5 kDa. After specific labeling, extract from DM2717 (pThiM3) contained two labeled polypeptides of approximately 27 and 29 kDa (Fig. 5, lane A). Extracts from similarly grown DM2718 (pThiM2), which contained the *thiM* and *thiD* ORFs in reverse orientation, lacked these two proteins (Fig. 5, lane B). As can be seen in Fig. 5, lane A, an additional protein, of approximately 40 kDa, was expressed from pThiM3. We believe that this band is an artifact of the construct, since it can be visualized by Coomassie staining under conditions where no inducing treatment has been performed. Taken together, these results supported the conclusion that the *thiM* and *thiD* genes encoded proteins of the predicted sizes.

PCR mapping and sequencing of insertions. To address phenotypic differences between insertion mutants, the locations of the two insertions described above and two additional insertions were determined by a PCR mapping procedure described previously (8, 41). The results of these experiments determined the locations of four insertion elements: three in ORF1 (*thiM*) and one in ORF2 (*thiD*) (Fig. 2). A 300-bp fragment was amplified between the Tn*10d*(Tc) and the MudJ insertion elements in DM3174 [*thiM937*::Tn*10d*(Tc) *thiM977*::MudJ], and a 700-bp product was amplified between the MuL and BR1 primers in DM176 (*thiM938*::MudJ), placing all three insertions in ORF1 (*thiM*). The ends of the amplified products were sequenced with primers specific to the insertion element, MuL (for DM176 and DM3174) and Tn10-I (DM3174), which confirmed the location (data not shown). A 200-bp product was generated by amplification between primers BF2 and MuL in strain DM456 (*thiD906*::MudJ), placing this insertion element in ORF2. Sequencing of the amplified fragment with the MuL primer confirmed this position. The map positions of the insertions were consistent with the phenotypic classes noted previously; e.g., strain DM456 was distinct from the strains containing the three remaining insertions.

***thiM* and *thiD* form an operon that is regulated by TPP.** The positions of the two genes in this *thi* locus suggested that they formed an operon. To address the possibility of polarity, the *thiD906*::MudJ insertion was transduced into DM1390 [*thiM937*::Tn*10d*(Tc)], generating isogenic strains DM3204 (*thiD thiM*) and DM3205 (*thiD*). To allow for pro-

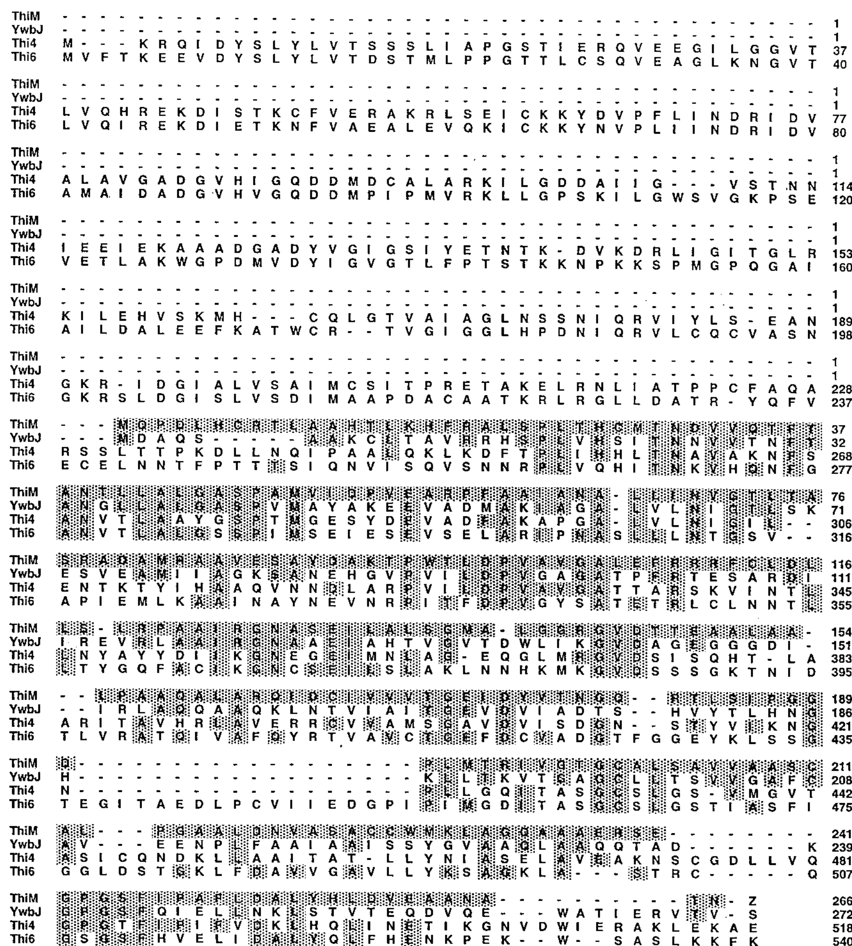

FIG. 4. ThiM is a hydroxyethylthiazole kinase. The DNASTAR alignment program was used to align protein sequences homologous to those of ThiM. *S. typhimurium* ThiM was homologous to the C-terminal end of Thi6 from *S. cerevisiae* and Thi4 from *S. pombe*. YwbJ from *B. subtilis* has recently been shown to possess THZ kinase activity and has been designated ThiK (42). Shaded regions represent amino acids that are completely conserved with respect to ThiM.

totrophic growth and to account for any involvement of these genes in their own transcription, plasmid pThiM1 was electroporated into both of the above strains and strain DM3169 (*thiM977*::MudJ). β-Galactosidase activity was measured in the three resulting strains, and results of a representative experiment are shown in Table 2.

Two conclusions can be drawn from the data presented in Table 2. First, transcription of both *thiM* and *thiD* is repressed in the presence of TPP. Table 2 shows a reduction of approximately threefold in transcription of a MudJ operon fusion in each gene in the presence of exogenous TPP. Second, insertions in the upstream *thiM* gene significantly reduced expression of the downstream *thiD* gene. This result was consistent with these two genes forming an operon and suggested that insertions in *thiM* exerted a polar effect on *thiD* that may be responsible for the phenotypic differences caused by insertions in these two genes. Strikingly, however, insertions in *thiM* caused a less severe phenotype than those in the downstream *thiD* gene.

One explanation for the distinct phenotypes was that *thiM* was nonessential and that reduced levels of *thiD* resulted in the phenotype associated with *thiM* insertions, i.e., leaky Thi$^-$ auxotrophy. A plasmid that contained only *thiM* was constructed to determine if the ThiM gene product was involved in the mutant phenotypes caused by insertions in *thiM*. Plasmid

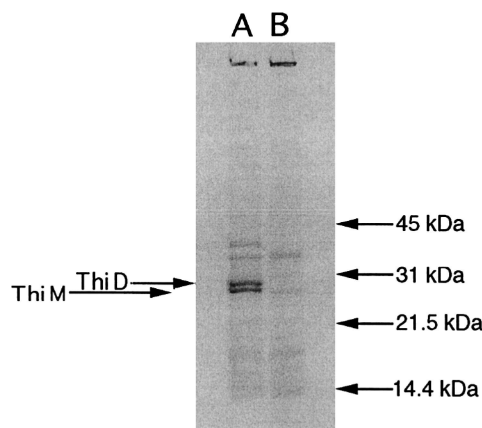

FIG. 5. Specific labeling of ThiM and ThiD. Strain DM2717(pThiM3) was used to label ThiM and ThiD with [^{35}S]methionine, and induction was performed by the method of Tabor (35). The T7 RNA polymerase was under the control of an IPTG-inducible promoter in *E. coli* BL21. Lane A contains extract from DM2717 and displays two proteins of 27 and 29 kDa. Lane B contains extract from control strain DM2718(pThiM2).

TABLE 2. *thiMD* comprises an operon repressed by TPP[a]

Strain	Genotype	β-Galactosidase activity (U at OD_{650})	
		No addition	With TPP
DM3171	*thiM977*::MudJ/pthiM1	60	20
DM3208	*thiD906*::MudJ *thiM937*::Tn*10d*/pThiM1	3	4
DM3209	*thiD906*::MudJ/pthiM1	59	18

[a] Strains were grown overnight in minimal gluconate medium containing chloramphenicol. A MudJ insertion results in an operon fusion. Aliquots of 0.1 ml were inoculated into 5 ml of the appropriate medium, and cultures were grown to an optical density at 650 nm (OD_{650}) between 0.3 and 0.4. All strains grew at similar rates, and β-galactosidase activity was assayed as described in Materials and Methods.

pThiM4 (Fig. 2) was constructed by ligating the *Nae*I/*Hin*cII fragment from pThiM1 into the *Sma*I site of pSU19. If the phenotypes caused by *thiM* insertions were due solely to polarity on the *thiD* gene, pThiM4 would be expected to have no effect on these phenotypes. No growth differences were detected when pThiM4 was electroporated into DM176 (*thiM938*::MudJ) and the resulting strain was compared with the parental strain (data not shown), indicating that phenotypes caused by *thiM* insertions were due to polarity on the *thiD* gene.

If insertions in *thiM* were completely polar on *thiD*, the phenotypes caused by an insertion in either gene should be identical. Because the growth defects caused by insertions in *thiD* were more severe than those caused by insertions in *thiM*, we suggested that a low level of *thiD* was responsible for the phenotypes associated with insertions in *thiM*. This reduced expression could be due to incomplete polarity of the MudJ insertion or to low-level promoter activity in front of *thiD.*

Reduced expression of *thiD* results in excretion of HMP. During the course of this work it was observed that strains carrying an insertion in *thiM* excreted a compound able to satisfy the requirement of HMP-requiring strains. It had been previously observed by others that certain mutants that are impaired for thiamine synthesis accumulate the HMP moiety and excrete it into the growth medium (18, 29). To determine if the biologically active compound was HMP, it was purified from 2 liters of DM176 (*thiM938*::MudJ) as described in Materials and Methods. Prior to HPLC purification, UV spectra were taken of the biologically active fraction at both pH 7.2 and pH 2.0. These spectra identified distinct λ_{max}'s of 270 and 245 nm, respectively. These pH-specific λ_{max}'s are characteristic of a pyrimidine moiety.

A concentrated sample of this fraction was applied to a C_{18} HPLC column and eluted with 15% acetonitrile–85% water. The biologically active compound had a retention time of 24 min and an A_{270} of 0.54. Spiking this sample with 2.5 nmol of authentic HMP (provided by Tadhg Begley, Cornell University) doubled the A_{270} of the peak at 24 min without any additional changes noted in the spectrum (data not shown). Further, the mass spectrum of an HPLC-purified sample identified a molecular ion of *m*/*z* 139, consistent with the molecular ion of HMP. The ^{1}H NMR spectrum of this sample in methanol identified signals at 2.4, 4.4, and 8 ppm, again consistent with the reported spectrum for HMP (4). Taken together, these data identified HMP as the biologically active compound excreted into the medium by strain DM176 (*thiM938*::MudJ).

As expected from these results, strain DM456 (*thiD906*::MudJ) excreted a similar compound when grown with limiting thiamine. This represented the first conclusive data showing that a block in the pyrimidine branch of thiamine synthesis can result in the excretion of HMP.

DISCUSSION

Work presented herein has increased our understanding of thiamine biosynthesis in *S. typhimurium*. Sequence data have clarified the physical structure of the *thiM* and *thiD* loci, previously defined by point mutations in *E. coli*. A 2-gene operon that encoded proteins of 27 and 29 kDa was identified. Based on strong sequence similarity to proteins with demonstrable thiazole kinase activity and phenotypic analyses, we designated the upstream gene *thiM*. The protein encoded by the 2nd gene in the operon had no homologs with known activity, and thus our designation of this gene as *thiD* was based on phenotypic analysis and map location. *thiD* mutants required the complete thiamine molecule for growth, as previously reported for *E. coli.*

Operon fusion experiments determined that the *thiMD* operon was regulated by thiamine and suggested that it contained an internal promoter to ensure expression of *thiD*. The ability of thiamine and TPP to repress transcription of *thiMD* made this the second operon involved in thiamine biosynthesis thus regulated. This is in contrast to two single-gene loci, *thiI* and *thiL*, which have been found not to be transcriptionally regulated by exogenous thiamine when an operon fusion is used (39, 40).

Finding polar insertions in *thiM* has fortuitously provided us with a tool to address aspects of thiamine synthesis that have been refractory to study, i.e., the synthesis of HMP from AIR. Results presented here demonstrated that an insertion in *thiM* resulted in significantly reduced expression of *thiD* and thus generated phenotypes distinct from those caused by the complete absence of *thiD*. Because this strain grew prototrophically under some conditions, we were able to determine that these mutants excreted HMP into the medium. Newell and Tucker observed that mutations disrupting the thiazole branch of the pathway led to the excretion of HMP, and they determined that the excretion was due to the derepression of the HMP branch caused by limiting THZ (27). The excretion of HMP documented here can be explained by reduced levels of ThiD limiting the conversion of HMP-P to HMP-PP, resulting in an accumulation of HMP-P, which is excreted as HMP. The excretion of HMP as opposed to the presumed substrate of ThiD (HMP-P) may indicate either that HMP is a direct precursor to HMP-P that can also accumulate or, alternatively, that an HMP-P dephosphorylating activity exists in the cell.

The identification of bradytrophic strains that excrete HMP provides us with a method for rapid purification of HMP for nutritional work and a means to identify mutations that disrupt this excretion. One class of these mutations should define biochemical steps in the formation of HMP, representing a set of genes that we and others have failed to identify by standard genetic screens. Further characterization of the phenotypes associated with strains limited for HMP-P kinase and the biochemical basis of these phenotypes will increase our understanding of thiamine synthesis in general and the conversion of AIR to HMP specifically.

ACKNOWLEDGMENTS

We thank Tadhg Begley for helpful discussion, providing data prior to publication, and supplying HMP.

This work was supported by NIH grant GM47296.

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. **Backstrom, A. D., R. Austin, S. McMordle, and T. P. Begley.** 1995. Biosynthesis of thiamin. I. The function of the *thiE* gene product. J. Am. Chem. Soc. **117:**2351–2352.
3. **Begley, T. P.** 1996. The biosynthesis and degradation of thiamine (vitamin F_1). Nat. Proc. Rep., p. 178–186.
4. **Begley, T. P.** 1997. Personal communication.
5. **Brown, G. M., and J. M. Williamson.** 1987. Biosynthesis of folic acid, riboflavin, thiamine, and pantothenic acid, p. 521–538. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
6. **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.
7. **Chen, R. K., T. Botstein, T. Watanabe, and Y. Ogata.** 1972. Specialized transduction of tetracycline resistance by phage P22 in *Salmonella typhimurium* II. Properties of a high transducing lysate. Virology **50:**883–898.
8. **Chen, P., M. Ailion, N. Weyland, and J. Roth.** 1995. The end of the *cob* operon: evidence that the last gene (*cobT*) catalyzes synthesis of the lower ligand of vitamin B_{12}, dimethylbenzimidazole. J. Bacteriol. **177:**1461–1469.
9. **David, S., B. Estramareix, J. C. Fischer, and M. Therisod.** 1982. The biosynthesis of thiamine. Synthesis of [1,1,1,5-$^2H_4$]-1-deoxy-D-*threo*-2-pentulose and incorporation of this sugar in biosynthesis of thiazole by *Escherichia coli* cells. J. Chem. Soc. Perkin Trans. I. **1:**2131–2137.
10. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. **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.
12. **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.
13. **Downs, D. M., and L. Petersen.** 1994. *apbA*, a new genetic locus involved in thiamine biosynthesis in *Salmonella typhimurium*. J. Bacteriol. **176:**4858–4864.
14. **Escalante-Semerena, J. C., and J. R. Roth.** 1987. Regulation of cobalamin biosynthetic operons in *Salmonella typhimurium*. J. Bacteriol. **169:**2251–2258.
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. **Imamura, N., and H. Nakayama.** 1981. *thiD* locus of *Escherichia coli*. Experientia **37:**1265–1266.
17. **Imamura, N., and H. Nakayama.** 1982. *thiK* and *thiL* loci of *Escherichia coli*. J. Bacteriol. **151:**708–717.
18. **Iwashima, A., K. Takahashi, and Y. Nose.** 1971. Overproduction of hydroxymethylpyrimidine by a thiamine regulatory mutant of *Escherichia coli*. J. Vitaminol. **17:**43–48.
19. **Kawasaki, T., and Y. Nose.** 1969. Thiamine regulatory mutants in *Escherichia coli*. J.Biochem. **65:**417–425.
20. **Kleckner, N., J. Bender, and S. Gottesman.** 1991. Uses of transposons with emphasis on Tn*10*. Methods Enzymol. **204:**139–180.
21. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Mizote, T., and H. Nakayama.** 1989. The *thiM* locus and its relation to phosphorylation of hydroxyethylthiazole in *Escherichia coli*. J. Bacteriol. **171:**3228–3232.
23. **Mizote, T., M. Tsuda, T. Nakazawa, and H. Nakayama.** 1996. The *thiJ* locus and its relation to phosphorylation of hydroxymethylpyrimidine in *Escherichia coli*. Microbiology **142:**2969–2974.
24. **Nakayama, H.** 1990. Genetic analyses of thiamine pyrophosphate biosynthesis in *Escherichia coli*. Vitamins **64:**619–632.
25. **Nakayama, H., and R. Hayashi.** 1972. Biosynthetic pathway of thiamine pyrophosphate: a special reference to the thiamine monophosphate-requiring mutant and thiamine pyrophosphate-requiring mutant of *Escherichia coli*. J. Bacteriol. **112:**1118–1126.
26. **Newell, P. C., and R. G. Tucker.** 1968. Biosynthesis of the pyrimidine moiety of thiamine. Biochem. J. **106:**279–287.
27. **Newell, P. C., and R. G. Tucker.** 1966. The control mechanism of thiamine biosynthesis. A model for the study of control of converging pathways. Biochem. J. **100:**517–524.
28. **Newell, P. C., and R. G. Tucker.** 1968. Precursors of the pyrimidine moiety of thiamine. Biochem. J. **106:**271–277.
29. **Newell, P. C., and R. G. Tucker.** 1970. Thiamine—methods for studying the biosynthesis of the pyrimidine moiety, p. 178–182. *In* D. B. McCormick and L. D. Wright (ed.), Vitamins and coenzymes, vol. 18A. Academic Press, Inc., New York, N.Y.
30. **Nosaka, K., H. Nishimura, Y. Kawasaki, T. Tsujihara, and A. Iwashima.** 1994. Isolation and characterization of the *thi6* gene encoding a bifunctional thiamin-phosphate pyrophosphorylase/hydroxyethylthiazole kinase from *Saccharomyces cerevisiae*. J. Biol. Chem. **269:**30510–30516.
31. **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.
32. **Petersen, L. A., J. E. 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.
33. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
34. **Schmieger, H.** 1972. Phage P22 mutants with increased or decreased transduction abilities. Mol. Gen. Genet. **119:**75–88.
35. **Tabor, S.** 1990. Expression using the T7 RNA polymerase/promoter system, p. 16.2.1–16.2.11. *In* F. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. C. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology. Wiley, New York, N.Y.
36. **Vander Horn, P. B., A. D. Backstrom, V. Stewart, and T. P. Begley.** 1993. Structural genes for thiamine biosynthetic enzymes (*thiCEFGH*) in *Escherichia coli* K-12. J. Bacteriol. **175:**982–992.
37. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218:**97–106.
38. **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.
39. **Webb, E., K. Claas, and D. Downs.** 1997. Characterization of *thiI*, a new gene involved in thiazole biosynthesis in *Salmonella typhimurium*. J. Bacteriol. **179:**4399–4402.
40. **Webb, E., and D. M. Downs.** 1997. Characterization of *thiL*, encoding thiamine monophosphate kinase, in *Salmonella typhimurium*. J. Biol. Chem. **272:**15702–15707.
41. **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.
42. **Zhang, Y., S. V. Taylor, H.-J. Chiu, and T. B. Begley.** 1997. Characterization of the *Bacillus subtilis thiC* operon involved in thiamine biosynthesis. J. Bacteriol. **179:**3030–3035.
43. **Zurlinden, A., and M. E. Schweingruber.** 1994. Cloning, nucleotide sequence, and regulation of *Schizosaccharomyces pombe thi4*, a thiamine biosynthetic gene. J. Bacteriol. **176:**6631–6635.