JOURNAL OF BACTERIOLOGY, Aug. 1994, p. 4858–4864
0021-9193/94/$04.00+0

Vol. 176, No. 16

apbA, a New Genetic Locus Involved in Thiamine Biosynthesis in *Salmonella typhimurium*

DIANA M. DOWNS* AND LESLIE PETERSEN

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

Received 3 February 1994/Returned for modification 14 April 1994/Accepted 3 June 1994

In *Salmonella typhimurium*, the synthesis of the pyrimidine moiety of thiamine can occur by utilization of the first five steps in de novo purine biosynthesis or independently of the *pur* genes through the alternative pyrimidine biosynthetic, or APB, pathway (D. M. Downs, J. Bacteriol. 174:1515–1521, 1992). We have isolated the first mutations defective in the APB pathway. These mutations define the *apbA* locus and map at 10.5 min on the *S. typhimurium* chromosome. We have cloned and sequenced the *apbA* gene and found it to encode a 32-kDa polypeptide whose sequence predicts an NAD/flavin adenine dinucleotide-binding pocket in the protein. The phenotypes of *apbA* mutants suggest that, under some conditions, the APB pathway is the sole source of the pyrimidine moiety of thiamine in wild-type *S. typhimurium*, and furthermore, the *pur* genetic background of the strain influences whether this pathway can function under aerobic and/or anaerobic growth conditions.

Thiamine (vitamin B_1) is a required nutrient for the cell and in its coenzymic form, thiamine pyrophosphate, participates as a carrier of C2 units in reactions such as the ones catalyzed by transketolase and pyruvate dehydrogenase. Thiamine consists of a pyrimidine moiety (4-amino-5-hydroxymethyl-2-methylpyrimidine [HMP]) and a thiazole moiety [4-methyl-5-(β-hydroxyethyl) thiazole], which are synthesized independently and later joined to form the functional vitamin. Significant work has focused on how these components are synthesized prior to being joined (11–13, 22–24, 32, 35–37). Early biochemical and genetic work indicated that the first five reactions of de novo purine biosynthesis are used by the cell to synthesize HMP. These five de novo purine biosynthetic reactions result in the formation of aminoimidazole ribotide (AIR), which can then be converted into imidazole monophosphate by *pur* gene products or into HMP by thiamine (*thi*) gene products (Fig. 1). The biochemical steps involved in the conversion of AIR to HMP have not been fully characterized, although a partial pathway has been proposed (3, 32).

Despite the fact that HMP can be derived from de novo purine biosynthesis, there is no evidence to suggest feedback or transcriptional control over AIR biosynthesis by thiamine. The *pur* genes are transcriptionally regulated by a common repressor protein, PurR, through levels of hypoxanthine (16, 25, 26). However, no regulation of *pur* transcription by thiamine has been observed (30a). In addition, while the first enzyme in de novo purine biosynthesis (PurF) is strongly inhibited by a variety of purines (20, 31), there is no report on whether its activity is affected by thiamine.

We have previously reported that *Salmonella typhimurium* can synthesize thiamine in the absence of a functional de novo purine biosynthetic pathway (8, 9). We showed that *purF* mutants can synthesize thiamine under certain growth conditions. For example, exogenous pantothenate in the medium can bypass a defective PurF in the synthesis of thiamine but does not allow detectable purine biosynthesis (9). It was proposed that the observed synthesis of thiamine was due to an alternative PurF function because under these conditions thiamine biosynthesis still required the remaining *pur* genes for the formation of HMP (9).

Recently, we demonstrated the existence of a pathway that can synthesize the pyrimidine moiety of thiamine in the absence of any *pur* genes involved in the formation of AIR (8). This pathway bypasses the requirement for the de novo purine biosynthetic genes to synthesize thiamine under anaerobic conditions. The pathway responsible for *pur*-independent thiamine synthesis is referred to as the alternative pyrimidine biosynthetic, or APB, pathway (8). We have thus far been unable to satisfy the cellular purine requirement through the APB pathway. There are several possible explanations for this. The relationship between the alternative PurF function postulated previously (9) and the APB pathway is unclear at present.

We report herein the initial genetic and physical characterization of the *apbA* locus. Our results are consistent with this locus encoding a structural gene in the APB pathway. The phenotypes of *apbA* mutants suggest that a functional APB pathway is required for the synthesis of thiamine when *S. typhimurium* is grown in the presence of exogenous purines.

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. MudJ is used throughout the manuscript to refer to the Mud dl1734 transposon, which has been described previously (4), and Tn*10*d(Tc) refers to the transposition-defective mini-Tn*10* (Tn*10*Δ-16 Δ-17) described by Way et al. (34). The E medium of Vogel and Bonner (33), supplemented with 11 mM glucose (aerobically) or 22 mM glucose (anaerobically), was used as minimal medium. 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 for aerobic growth, and Acumedia agar (Acumedia, Baltimore, Md.), added to achieve the same concentration, was used for anaerobic growth. When present in the culture media, and unless otherwise stated, the final concentrations of the compounds were as follows: adenine, 5 mM; thiamine, 50 μM; pantothenate, 50 μM. The final concentrations of the antibiotics in rich medium were as follows: tetracycline, 20 μg/ml; kanamycin, 50 μg/ml; ampicillin, 30 μg/ml; chloramphenicol,

* Corresponding author. Mailing address: Department of Bacteriology, 1550 Linden Dr., Madison, WI 53706. Phone: (608) 265-4650. Fax: (608) 262-9865. Electronic mail address: Downs@vms2.macc.wisc.edu.

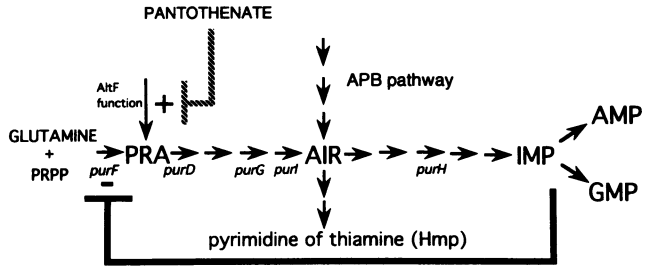

FIG. 1. Pathway schematic. The purine and APB pathways are illustrated. The gene products required for selected reactions are indicated under the relevant arrow. The known regulation by purines of the function of the PurF gene product is indicated with a solid line. The proposed activation of the alternative PurF function by pantothenate (9) is also indicated, with a shaded line. The number of steps involved in the formation of the pyrimidine moiety of thiamine from AIR is not known. The conversion of AIR to the first complete purine requires the action of five de novo purine genes. Abbreviations: PRPP, phosphoribosyl pyrophosphate; PRA, phosphoribosyl amine.

20 μg/ml. The concentrations of these antibiotics in minimal medium were 10, 125, 15, and 4 μg/ml, respectively.

Growth curves. Growth curves were done as follows. Cells from a full-density nutrient broth culture were pelleted and suspended in the original volume of 0.85 M NaCl. A 1:25 inoculation was made into the indicated medium. Cultures were then incubated at 37°C with (aerobic) or without (anaerobic) shaking, and turbidity was monitored with a Bausch & Lomb Spectronic 20 spectrophotometer at an optical density of 650 nm. The specific growth rate, μ, was defined as $\ln(X_0/X)/T$.

Genetic techniques. Transduction methods. The high-frequency general transducing mutant of bacteriophage P22 (HT105/1, *int-201*) (28) was used in all transductional crosses. Recipient cells (ca. 10^8 CFU) and transducing phage (ca. 10^8 to 10^9 PFU) were preincubated for 1 h at 25°C without shaking prior to plating on selective medium. When tetracycline resistance (Tc^r) or prototrophic growth was the basis for selection, the cells and phage were spread directly on selective plates and incubated at 37°C. Transductants were purified by streaking on nonselective green indicator plates, and putative phage-free clones were identified on the basis of their light-colored colonies (5). Possible phage-free colonies were checked for phage sensitivity by cross-streaking with phage P22.

TABLE 1. Bacterial strains[a]

Strain	Genotype	Source
LT2	Wild type	Laboratory strain
DM63	*apbA1*::MudJ[b]	
DM77	*apbA1*::MudJ *purF2100 purG1739*::Tn*10*	
DM86	*zba-8007*::Tn*10*d[c]	Laboratory strain
DM129	*purF2100 purG1739*::Tn*10*	
DM171	*apbA* Δ*8*	
DM185	*apbA1*::MudJ, pAPB1	
DM186	*apbA* Δ*6*	
DM187	*apbA1*::MudJ, pAPB2	
DM197	*apbA1*::MudJ, pAPB3	
DM219	*zaj-1034*::Tn*10*	
DM254	DH5α (*E. coli*)/pAPB4	
DM255	DH5α (*E. coli*)/pAPB5	
DM269	*thi-1887*::Tn*10*	Laboratory strain
DM368	*apbA1*::MudJ, pAPB8	
DM370	*zba-8007*::Tn*10*d *apb11*	
DM371	*zba-8007*::Tn*10*d *apb12*	
DM372	*cobA367*::Tn*10*d(Tc), pGP1-2, pAPB6	
DM373	*cobA367*::Tn*10*d(Tc), pGP1-2, pAPB7	
TT10288	*hisD9953*::MudJ *his-9944*::Mud1	J. Roth
TT15235	*purE2154*::MudQ	J. Roth
JE2017	*cobA367*::Tn*10*d(Tc), pGP1-2	J. Escalante-Semerena

[a] Unless indicated otherwise, strains were constructed for this study.
[b] MudJ is used throughout the text to refer to the Mud dl1734 transposon (4).
[c] Tn*10*d refers to the transposition-defective mini-Tn*10* (Tn*10*Δ-16 Δ-17) (34).

Mutagenesis. (i) Transposon insertion. Transducing phage was grown on a strain (TT10288) carrying MudJ insertions *hisD9953*::MudJ and *his-9944*::Mud1. In this strain the Mud1 supplies transposase functions, allowing the transposition-deficient MudJ to transpose with high frequency when both are introduced into a new strain (15). This lysate was used to transduce a strain defective in purine biosynthesis (DM129, *purF2100 purG1739*::Tn*10*) to kanamycin resistance (Km^r) on minimal plates containing adenine and reduced levels of thiamine (50 nM). The transduction plates were replica plated to minimal plates containing adenine and kanamycin with or without thiamine. These plates were then incubated in an anaerobic hood (Coy type) at 37°C overnight and scored for Thi^- clones, which were further characterized. Subsequent screenings for mutants were done aerobically, by screening for an adenine sensitivity which could be corrected by either thiamine or pantothenate. Insertions of the Tn*10*d(Tc) element in *apbA* were isolated from a pool of strains (100,000), each containing one Tn*10*d(Tc) element randomly inserted in the chromosome (17).

(ii) Point mutations. Point mutations in *apbA* were generated by growing a P22 lysate on a strain containing a Tn*10*d(Tc) insertion >90% linked to the *apbA* locus (DM86). This lysate was mutagenized with hydroxylamine as previously described (6, 14) and used to transduce wild-type *S. typhimurium* LT2 to Tc^r. Tc^r transductants were then screened aerobically for adenine sensitivity.

(iii) Deletion mutations. Deletion mutations were generated by plating strain DM86 on Bochner plates (2) as modified by Maloy et al. (18). Resulting Tc^s clones were screened for those which were *apbA* mutants.

Mapping *apbA*. The *apbA* locus was mapped by the method of Benson and Goldman (1). Induced lysates of strains carrying Mud-P22 insertions around the chromosome were spotted on a minimal adenine plate seeded with P22 tail protein and an *apbA* recipient strain (DM63). Map location was approximated by the chromosomal position of the Mud-P22 in lysates able to repair the *apbA* mutation. Further mapping was done by determining P22 linkage of *apb* mutations to nearby markers.

β-Galactosidase assays. The cleavable substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (at 20 μg/ml) was used as a colorimetric indicator of β-galactosidase activity in agar plates. β-Galactosidase was assayed by the method of Miller (21) as reported previously (10).

Recombinant DNA techniques. (i) Cloning. Standard methods were used for DNA restriction endonuclease digestion and ligation. Restriction enzymes and ligase were purchased from New England Biolabs (Beverley, Mass.). Plasmid DNA was isolated with the Magic Mini-preps system purchased from Promega (Madison, Wis.) and was transferred between strains by electroporation using a BioRad (Richmond, Calif.) E. Coli Pulser. *Escherichia coli* DH5α was used as a recipient during some plasmid construction steps.

(ii) DNA sequencing. DNA sequences were determined from denatured double-stranded templates by the dideoxynu-

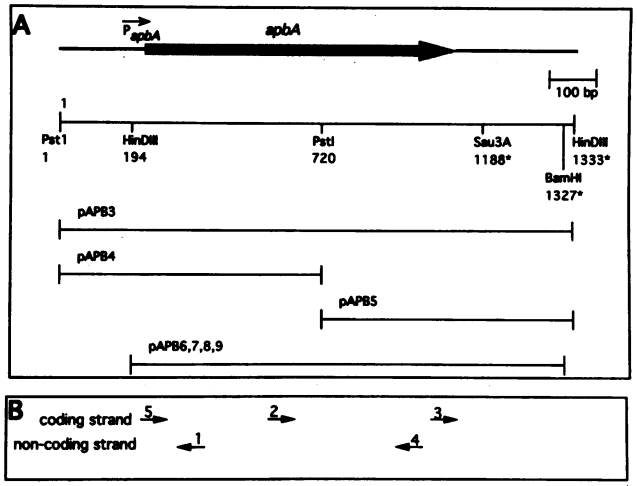

FIG. 2. Plasmids and primers used to sequence *apbA*. Shown at the top of the figure is a schematic with the *apbA* coding sequence in boldface and the flanking regions indicated with thin lines. The presumed site of the *apbA* promoter is represented, and the orientation of the ApbA ORF is indicated by the arrowhead. Directly below is a partial restriction map showing the relevant sites used to generate subclones and their positions relative to nt 1 on the left. The sequence presented (Fig. 5) is equivalent to nt 158 to 1188 in this restriction map. The *Sau*3A site at position 1188 (*) is a junction of DNA which is noncontiguous on the chromosome, and thus sequences past this point were not considered in any analyses. (A) The inserts of subclones of pAPB3 used for sequencing and overexpression are shown. The ends of the inserts are aligned with the restriction sites (top of figure) used to generate them. (B) Internal primers used to sequence and their positions are indicated, as is assignment to either the coding or noncoding strand. In addition, manufacturers' primers were used for sequencing the ends of clones pAPB3, pAPB4, and pAPB5. The −40 primer was used to read the coding strand in from the left of the figure, and the reverse primer was used to read the noncoding strand in from the right.

cleotide chain termination method (27) with modified T7 DNA polymerase (Sequenase; United States Biochemicals, Cleveland, Ohio) and [^{35}S]dATP (specific activity, 1,000 to 1,500 Ci/mmol) (Dupont, Beverly, Mass.). Oligonucleotides were purchased from United States Biochemicals (−40 and reverse primer for M13mp19) or generated by National Biosciences (Plymouth, Minn.) complementary to sequences internal to *apbA*. All reactions were carried out as per manufacturers' specifications, both DNA strands of the *Pst*I-*Hin*dIII insert of pAPB3 were sequenced in their entirety at least twice independently, and the gels were read at least three times. Initially, the inserts of pAPB4 and pAPB5 were sequenced with the manufacturers' primers (−40 reading the coding strand and the reverse primer reading the noncoding strand); additional primers (18 nucleotides [nt]) were designed to complete the sequence. The location of these primers is illustrated in Fig. 2. These primers were used to sequence across the junction in pAPB3 to confirm the subcloning. The sequences of the primers are as follows: primer 1, 5′ TGC GTA TCG ATC AGA TT 3′; primer 2, 5′ CGC GAC GGT AAT ATC AT 3′; primer 3, 5′ AAA TTG ACT ACA TCA CC 3′; primer 4, 5′ CGA ACA TGG ATG AGA TG 3′; primer 5, 5′ GTG AGG AGG AAC AAT GA 3′.

Plasmids. The inserts of the following plasmids are shown in Fig. 2. For pAPB1 (not shown), a partial *Pst*I digest of DNA generated by inducing the *purE*::MudP22 from strain TT15235 was ligated into pSU19 (previously cut). pAPB2 (not shown) was generated by dropping out a *Hin*dIII fragment from pAPB1 and religating (this dropped-out fragment involved the *Hin*dIII site at nt 1333 in Fig. 2 and the *Hin*dIII site in the multiple cloning site (MCS) of pSU19). pAPB3 was generated by dropping out a *Sau*3A fragment to generate a join point which is not contiguous on the chromosome. To accommodate this, the end of the insert sequence was treated as nt 1188, the regenerated *Sau*3A site. pAPB4 was generated by ligating a *Pst*I fragment from pAPB3 into pSU19 (previously digested). pAPB5 was generated by ligating the *Pst*I-*Hin*dIII fragment from pAPB3 into pSU19 (previously digested). pAPB6, pAPB7, pAPB8, and pAPB9 were generated by ligating the *Hin*dIII-*Bam*HI fragment from pAPB3 into pT7-6, pT7-5, pSU18, and pSU19, respectively, which had been previously digested.

Overexpression and visualization of ApbA. A promoterless *Hin*dIII-*Bam*HI fragment from pAPB3 was cloned into T7 overexpression vectors pT_7-6 and pT_7-5, which differ only in the orientation of their MCS with respect to the T7 promoter (30). The resulting plasmids (pAPB6 and pAPB7) were electroporated into a *S. typhimurium* strain (JE2017) which carries a plasmid encoding T7 polymerase under the control of a heat-inducible promoter (pGP1-2). Specific labeling was done in vivo according to the coupled T7 RNA polymerase-T7 promoter method (30). The ApbA protein expressed from pAPB6 was labeled with [^{35}S]methionine and analyzed by sodium dodecyl sulfate (0.1%)-polyacrylamide gel electrophoresis (12% polyacrylamide) (SDS-PAGE) using the Bio-Rad MiniProtean electrophoresis system, and the protein products were visualized by autoradiography with Kodak Direct Exposure film.

Computer analysis. The DNA sequence was analyzed by the Genetics Computer Group (Madison, Wis.) DNA sequence analysis program (7).

Nucleotide sequence accession number. The nucleotide sequence of *apbA* shown in Fig. 5 has been deposited in GenBank with the accession number U09529.

RESULTS

Isolation of *apbA* mutants. Our previous work had shown that *pur* mutants blocked prior to the formation of AIR were capable of synthesizing the pyrimidine moiety of thiamine under anaerobic conditions (8). This ability was attributed to the APB pathway.

We isolated *apb* derivatives of a *purF2100 purG1739*::Tn*10* mutant (DM129) by screening for MudJ (Km^r) insertion mutations that rendered this strain unable to grow on adenine under anaerobic growth conditions unless provided with exogenous thiamine. The screening of approximately 20,000 Km^r transductants resulted in four mutants which had an anaerobic as well as an aerobic thiamine auxotrophy. These mutants were analyzed further to distinguish between *apb* mutants and mutants defective in the *thi* genes. The MudJ insertion from each of the putative mutants was moved by P22 transduction into the parent strain DM129 and into a *pur*$^+$ strain (LT2) by selecting for Km^r. The phenotypes of these resulting strains were tested aerobically and anaerobically. We expected that *apb* mutants would be phenotypically silent in the *pur*$^+$ background due to the production of AIR through de novo purine synthesis (23, 24). Only one of the four MudJ elements was inserted into an *apb* locus. That is, when this insertion was introduced into DM129 (*purG purF* double mutant), the strain was auxotrophic for thiamine both aerobically and anaerobically. However, when the MudJ element was introduced into a

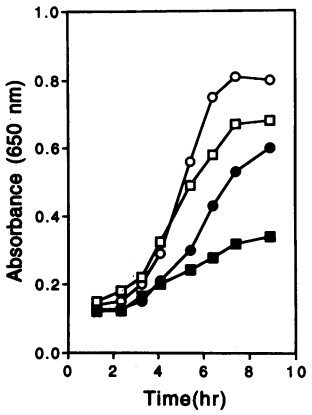

FIG. 3. Anaerobic growth of *purF purG apbA* mutants. Cultures were grown in standing tubes as described in Materials and Methods. Growth was carried out in a 37°C water bath. Filled symbols represent growth of DM77 (*apbA1*::MudJ *purF2100 purG1739*::Tn*10*), and open symbols represent growth of the parent strain, DM129 (*purF2100 purG1739*::Tn*10*). Squares represent minimal medium supplemented with 5 mM adenine, and circles represent minimal medium supplemented with 5 mM adenine and 50 μM thiamine.

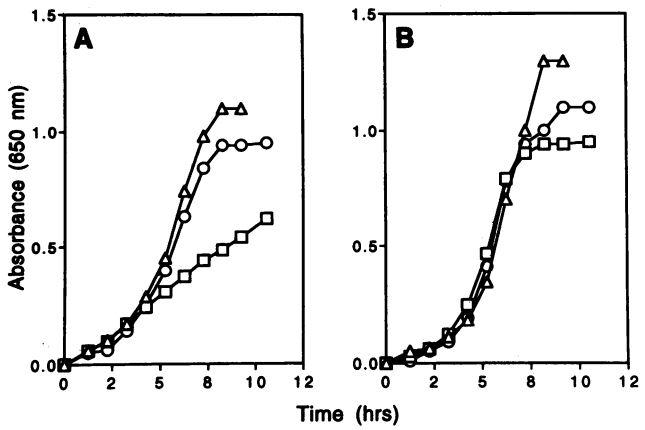

FIG. 4. Aerobic thiamine requirement of *apbA* mutants. Cultures were grown in a 37°C water bath with shaking as described in Materials and Methods. (A) Growth of DM63 [*apbA1*::Tn*10*d(Tc)] in minimal medium (△), minimal medium with 5 mM adenine (□), and minimal medium with 5 mM adenine and 50 μM thiamine (○). (B) Growth of LT2 (wild type) in minimal medium (△), minimal medium with 5 mM adenine (□), and minimal medium with 5 mM adenine and 50 μM thiamine (○).

pur$^+$ strain, the resulting strain (DM63) was prototrophic (i.e., grew on minimal medium). This result indicated that this insertion was not affecting any *thi* gene required to convert AIR to the pyrimidine moiety of thiamine. This mutation defined the first *apb* locus and is hereafter referred to as *apbA1*::MudJ.

The effect of the *apbA1*::MudJ mutation on the growth behavior of the *purF purG* double mutant under anaerobic conditions is illustrated in Fig. 3. As can be seen in this figure, DM77 (*apbA purF purG*) was defective in thiamine synthesis compared with its parent strain, DM129 (*apb*$^+$). This is illustrated by the inability of DM77 to grow in medium containing adenine alone. The minor amount of growth seen with DM77 in adenine medium was attributed to the carryover of thiamine from the inoculating culture. This conclusion was based on control experiments performed with a *thiC* mutant (data not shown). We have subsequently isolated a variety of mutations in this locus by various local mutagenesis techniques (see Materials and Methods). All the *apbA* mutants we have isolated display the same growth phenotype. Thus, mutants with mutations in this locus meet the criterion for being defective in the APB pathway.

Phenotypic characterization of *apbA* mutants in a *pur*$^+$ background. During the course of the genetic reconstructions described above, an unexpected phenotype of the *apbA* mutants was observed. A *pur*$^+$ *apbA* mutant (DM63) required thiamine under both aerobic and anaerobic growth conditions whenever adenine was present in the culture medium (Fig. 4). This suggests a requirement for the APB pathway to synthesize thiamine in wild-type strains grown in medium containing purines. Strain DM63 grew at wild-type rates in minimal medium as well as in medium that was supplemented with adenine and thiamine (μ = 0.4). In the presence of adenine alone, however, the growth rate of this strain dropped to one-half (μ = 0.2 [Fig. 4A]). The wild-type strain (LT2) showed no significant difference in growth rate in all three media (μ = 0.46 [Fig. 4B]). Other alleles of the *apbA* locus showed similar growth behavior in a *pur*$^+$ genetic background. This adenine-sensitive phenotype was severe enough to prevent growth on solid minimal medium containing adenine and was used to screen mutants in subsequent searches for APB pathway-defective strains as well as the isolation of a complementing clone (see below). It should be noted that only a small number (1/10) of mutants identified as adenine-sensitive met the criterion for *apb* mutants, that is, causing an anaerobic thiamine auxotrophy in *pur* strains.

Genetic characterization of the *apbA* locus. The location of the *apbA1*::MudJ element was genetically mapped between 8.5 and 12 min on the *S. typhimurium* chromosome by the method of Benson and Goldman as described in Materials and Methods (1). Subsequently it was shown by P22 transduction that *apbA* was 50% linked to *zaj-1034*::Tn*10* and 50% linked to *thiI887*::Tn*10*.

Transcriptional regulation. The insertion in strain DM63 (*apbA1*::MudJ) was judged by its color on X-Gal indicator plates (see Materials and Methods) to be in the correct orientation for expression of the *lacZ* gene from the *apbA* promoter. We assayed β-galactosidase produced from this fusion under a variety of growth conditions to assess possible transcriptional regulation of this gene. The assays were performed in two strains. DM63 contained only the *apbA1*::MudJ insertion. A second strain (DM197) contained the *apbA1*::MudJ insertion in the chromosome but carried a plasmid (pAPB3) which contains a wild-type copy of *apbA* cloned into the mid-copy-number vector pSU19 (19). This strain was used to ensure availability of ApbA in case *apbA* expression was autoregulatory. Neither strain showed any significant transcriptional regulation by adenine, histidine, thiamine, pantothenate, or oxygen. Transcription of the *apbA*::MudJ fusion remained between 20 and 35 units (10) under all tested conditions.

Sequencing of *apbA*. A preparation of DNA enriched for the region surrounding *apbA* was obtained by inducing a *purE*::Mud-P22 locked-in prophage (1) (strain TT15235) by the method of Youderian et al. (38) modified as described in Materials and Methods. This DNA was subjected to partial digestion with *Pst*I and ligated into the MCS of plasmid pSU19 which had been previously digested. The ligation mixture was electroporated into strain DM63 (*apbA1*::MudJ), and inheritance of plasmids was selected for on the basis of resistance to chloramphenicol (Cm^r). The Cm^r isolates were screened for

```
 1 AGCGCGGTTTTTGGCTCCCGCCCCTGCTCGCGGCAAAGCTTAGCTGATGACGCTATAATT  60
                    -35apbA                  -10apbA
61 AGCGCCATAACACCTGGCGCTGTTGCAGGTGAGGAGGAACAATGAAAATTACTGTACTGG 120
                              SDapbA     M K I T V L G

121 GATGCGGAGCCTTAGGACAACTTTGGCTTTCGGCACTGTGCAAACACGGACATGACGTAC 180
     C G A L G Q L W L S A L C K H G H D V Q

181 AGGGCTGGCTACGGGTGCCACAACCTTATTGCAGCGTAAATCTGATCGATACCGATGGTT 240
     G W L R V P Q P Y C S V N L I D T D G S

241 CCTTTTTCAACGAATCACTGACGGCAAACGATCCGGATTTTTTGGCGAAGAGCGAACTGT 300
     F F N E S L T A N D P D F L A K S E L L

301 TATTAGTCACGCTGAAAGCAGGTTTCCGACGCAGTACGAACCCTGGCGTCAACCTACCGG 360
     L V T L K A G F R R S T N P G V N L P V

361 TGACGTCGCCTATCCTGCTCATTCATAATGGAATGGGAACCATAGAAGAACTGCAAAATA 420
     T S P I L L I H N G M G T I E E L Q N I

421 TCCAACAGCCGATGTTGATGGGGACTATCACCCATGCTGCGCGCGACGGTAATATCATTA 480
     Q Q P M L M G T I T H A A R D G N I I I

481 TTCATGTCGCTAACGGTACTACGCATATTGGCCCGGCGCGCGAGCAAGACGGCGATTACA 540
     H V A N G T T H I G P A R E Q D G D Y S

541 GTTATCTCGCCGATATCCTGCAGGGGGTACTGCCCGATGTCGCCTGGCATAACAATATCC 600
     Y L A D I L Q G V L P D V A W H N N I R

601 GCGCAGAGATGTGGCGCAAACTGGCGGTCAACTGTGTTATCAACCCGCTCACCGCGCTTT 660
     A E M W R K L A V N C V I N P L T A L W

661 GGAACTGCCCGAACGGCGAGCTGCGCCATCATACTGATGAAATCAACGCGATTTGCGAAG 720
     N C P N G E L R H H T D E I N A I C E E

721 AGGTCGCCGCCGTGATTGAACGTGAAGGCTACCACACATCGGCAGACGATTTACGCTATT 780
     V A A V I E R E G Y H T S A D D L R Y Y

781 ATGTTGAGCAAGTGATTGACAGTACGGCAGAAAACATCTCATCCATGTTCGAGGACGTGC 840
     V E Q V I D S T A E N I S S M F E D V R

841 GTGCCATGCGCCATACCGAAATTGACTACATCACCGGTTATTTACTCAAGCGCGCCCGCG 900
     A M R H T E I D Y I T G Y L L K R A R V

901 TGCACGGTCTCGCGTTCCGGAAAATAGCCGCCTGTTTGAAATGGTAAAGCGAAAGGAGAG 960
     H G L A F R K I A A C L K W *

961 TGAGTATGAGCGCTCAGGCACTGGTATGCCTCGCCCCTGGTAGTGAAGAGACCGAAGCGG 1020

1021 TCACCACTAT 1030
```

FIG. 5. Nucleotide sequence of *apbA*. The nucleotide sequence of *apbA* was determined by sequencing with the chain-terminating method using Sequenase version 2.0 as described in Materials and Methods. Both strands were sequenced completely at least twice. The nucleotide sequence was analyzed with the Genetics Computer Group (Madison, Wis.) sequence analysis program (7). The nucleotide residues for −10 and −35 regions of the putative *apbA* promoter and the Shine-Dalgarno sequence are underlined. The asterisk represents the stop codon.

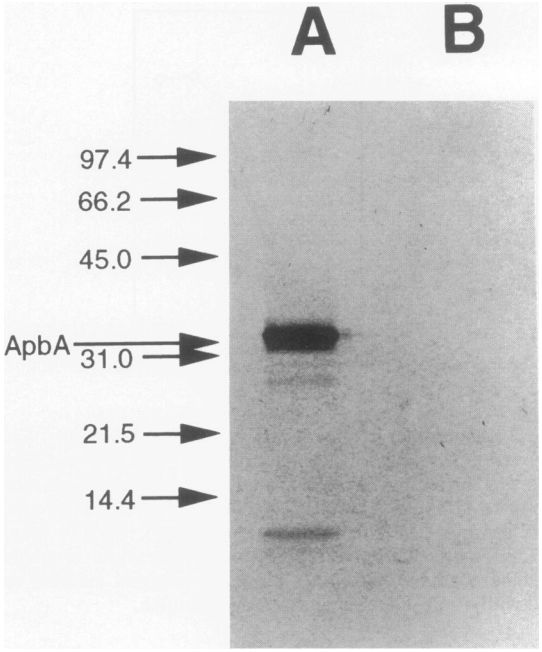

FIG. 6. Labeling of ApbA. Strain JE2017 [*cobA367*::Tn*10*d(Tc)] was used to label ApbA with [^{35}S]methionine, and assays were performed by the method of Tabor (30). The T7 RNA polymerase was provided in *trans* on pGP1-2. With this method, only the protein(s) expressed from the T7 promoter incorporates the [^{35}S]methionine. The crude extracts were analyzed by 0.1% SDS–PAGE (12% polyacrylamide), and proteins were visualized by autoradiography. The low-M_r protein ladder was purchased from Bio-Rad, and the sites of migration of the size markers are denoted with arrows. Strain JE2017 was transfected with pAPB6 (*Hin*dIII-*Bam*HI fragment in pT7-6) (lane A) and pAPB7 (*Hin*dIII-*Bam*HI fragment in pT7-5) (lane B). The Coomassie blue stain indicated that approximately equal amounts of protein were loaded in each lane. ApbA is visible only in lane A, migrating with an apparent molecular size of 32.5 kDa.

the presence of an *apbA*$^+$-complementing clone on the basis of growth on minimal plates containing adenine and chloramphenicol. One such clone (pAPB1) was isolated and used in further work. Plasmid pAPB1 contained a 6-kb fragment and was further subcloned to obtain a 1.3-kb *Pst*I-*Hin*dIII fragment in pSU19 (pAPB3) which complemented all *apbA* mutations tested. The inserts contained in each subsequent plasmid in the subcloning are illustrated in Fig. 2. The 1.3-kb *Pst*I-*Hin*dIII fragment from pAPB3 was sequenced in its entirety on both strands by the chain termination method of Sanger et al. as described in Materials and Methods (27). This was accomplished by first subcloning the fragment into pSU19, resulting in two plasmids (pAPB4 and pAPB5), each of which was sequenced from both ends with the manufacturer-provided −40 and reverse primers for M13mp19. Additional internal primers (described in Materials and Methods) were generated to complete the sequence on both strands as well as across the junction of the two subclones in pAPB3.

We found one open reading frame (ORF) of significant size (846 bp) in this sequence, which we tentatively assigned as *apbA*. The sequence of the ORF and its putative promoter and Shine-Dalgarno sequence are shown in Fig. 5. Our genetic data support the designated sequence as the promoter since a subclone of this insert cleaved at the *Hin*dIII site at base pair 36 complements an *apbA* mutant only if a promoter is provided in the vector (pAPB9). Plasmid pAPB9 allows growth of an *apbA* mutant on adenine and in addition restores the ability of a *purF purG apbA* mutant to grow anaerobically with adenine in the absence of thiamine (data not shown). On the basis of these results we predict ApbA to be a protein of 282 amino acids and 31.3 kDa in mass.

Overexpression of ApbA. In order to confirm our assignment of the ORF to the *apbA* gene, we cloned the promoterless *Hin*dIII-*Bam*HI fragment from pAPB3 into the T7 overexpression vectors pT7-6 and pT7-5, generating pAPB6 and pAPB7, respectively (30) (Fig. 2). These two clones were electroporated into JE2017, an *S. typhimurium* strain containing plasmid pGP1-2, which encodes T7 RNA polymerase. This generated strains DM372 and DM373, which were then used for specific labeling with [^{35}S]methionine (30) (see Materials and Methods). Proteins in crude cell extracts were resolved by SDS-PAGE, and labeled proteins were visualized by autoradiography.

Representative results of such a labeling experiment are shown in Fig. 6. Lane 1 [extract from DM372(pAPB6)] contains a single labeled polypeptide of approximately 32.5 kDa, consistent with the 31.3 kDa expected from the predicted amino acid sequence. Lane 2 contains extract of DM373, which contains the *apbA* ORF in reverse orientation and shows no labeled protein. This result is consistent with the genetic data

FIG. 7. Conserved primary structure motif in the ApbA protein. Primary structures of the dinucleotide binding sites of various known dehydrogenases are compared with the N-terminal sequence of the ApbA protein. All enzymes listed require an NAD cofactor. Abbreviations: AlgD, GDP–mannose 6-dehydrogenase (*Pseudomonas aeruginosa*); GADPH, glyceraldehyde 3-phosphate dehydrogenase (*Trypanosoma brucei*); TerA, terpredoxin reductase (*Pseudomonas* sp.); ProC, pyrroline-5-carboxylate reductase (*E. coli*); BaiH, NADH-dependent oxidoreductase (*Proponibacteriaceae*); FTDH, formyltetrahydrofolate dehydrogenase (rat). Most highly conserved residues are indicated: Δ, hydrophilic; ●, hydrophilic; |, obligate glycine residues.

described above which indicate that the promoter of *apbA* spans the *Hin*dIII site at bp 36, and no ApbA protein is expressed from this fragment in the absence of a promoter in the vector.

Computer analysis. The putative ApbA protein showed all the highly conserved residues of an NAD/flavin adenine dinucleotide (FAD)-binding motif (for examples, see Fig. 7). The strongest similarity was found to a family of NAD-dependent oxidoreductases from both procaryotes and eucaryotes.

DISCUSSION

We have isolated mutations in a locus required for the synthesis of thiamine via the *pur*-independent APB pathway. These mutations define the *apbA* locus and map to 10.5 min on the *S. typhimurium* chromosome. Mutations in this locus prevent anaerobic synthesis of the pyrimidine moiety of thiamine in a strain blocked for the synthesis of AIR via the de novo *pur* genes. Thus, by definition these mutants block the function of the APB pathway (8). *apbA* mutants were distinguished from *thi* mutants by their ability to synthesize thiamine when provided with a functional de novo purine pathway, indicating that they do not affect the thiamine-specific synthetic genes.

The *apbA* gene does not appear to be transcriptionally regulated under the conditions we tested. Experiments to address other possible modes of regulation of this gene are under way.

The *apbA* sequence predicts a NAD/FAD-binding pocket in the protein on the basis of the presence of all conserved residues for such a motif. This result is consistent with *apbA* encoding a structural gene in the APB pathway. In addition, it suggests that our future work to define the enzymatic activity of ApbA should include monitoring NAD/FAD redox biochemistry.

Mutants defective in *apbA* in an otherwise wild-type background (i.e., *pur*$^+$) require thiamine when grown in the presence of adenine (5 mM) either aerobically or anaerobically. This phenotype would not expected if the APB pathway were simply a redundant pathway for the synthesis of AIR. This result increases our understanding of the physiological role of the APB pathway in *S. typhimurium* by illustrating two points. First, the APB pathway can function aerobically, a result not predicted by the previous work carried out in *pur* mutant genetic backgrounds. Second, in wild-type *S. typhimurium*, in the presence of adenine, the synthesis of thiamine is affected, directly or indirectly, by the APB pathway in both aerobically and anaerobically grown cells. The results described above emphasize the significance of the APB pathway in the metabolism of *S. typhimurium*, particularly since, in the primary natural environment for this bacterium (i.e., the gut), purines are almost certainly present at concentrations at or above 5 mM (29).

The ability of this pathway to function aerobically, though previously thought not to occur (8), appears to depend on the genetic background of the strain. We, as well as others, have been unable to demonstrate that *pur* insertion mutants blocked in the conversion of phosphoribosyl amine to AIR (*purG*, *purD*, *purI*) can synthesize thiamine aerobically. This result indicates that in this genetic background the APB pathway does not function aerobically. However, lack of oxygen seems to compensate for the missing *pur* genes, since these strains can synthesize thiamine anaerobically. We have been unable, even after extensive mutagenesis, to isolate gain-of-function derivatives of mutants containing insertion mutations in these genes capable of aerobic synthesis of thiamine. This result suggests that the lack of APB pathway function aerobically in this genetic background is due to more than a single regulated step.

In contrast, the phenotype of our *apbA* mutants (Pur$^+$) shows that, in a strain which is competent for de novo purine synthesis but inhibited (i.e., by feedback inhibition of the first enzyme), the APB pathway is functional aerobically. This is indicated by the thiamine auxotrophy "induced" by the presence of exogenous adenine. Other results demonstrate that the APB pathway can also function aerobically if *purF* alone is defective and/or if enzymatic steps subsequent to the formation of AIR (i.e., *purH*) are blocked (unpublished results). Additional data suggest that the APB pathway activity is detectable aerobically in some *purG* and *purI* point mutants (13a). This raises the possibility that some of the *pur* gene products involved in the conversion of phosphoribosyl amine to AIR, or their substrates, are involved in the regulation and/or function of the APB pathway. Even though we can prevent carbon flow through the de novo pathway with a *purF* deletion, we cannot completely rule out the involvement of purine biosynthetic metabolites in APB regulation until we eliminate the alternative PurF function.

One explanation of the thiamine requirement of *apbA* mutants only in the presence of purines is that the exogenously added purines allosterically inhibit the PurF enzyme (glutamine amidotransferase), as they are known to do in vitro (20, 31). This might affect carbon flow through the de novo purine pathway, resulting in a thiamine auxotrophy if the compensatory pathway to make thiamine under these growth conditions (i.e., the APB pathway) is blocked. Such a model predicts that bypassing the PurF enzyme would relieve the thiamine auxotrophy of an *apbA* mutant "induced" by adenine. We have previously shown that exogenous pantothenate bypasses the requirement for the PurF enzyme in the synthesis of thiamine (9). The mechanism of this bypass is unknown but is postulated to be due to the activation of an alternative PurF function (9) (unpublished results). We found that exogenous pantothenate satisfied the thiamine requirement of our *apbA* mutants when adenine was present. This result is consistent with the hypothesis that the inability of *apbA* mutants to grow in the presence of purines is due to feedback regulation of the PurF enzyme, preventing carbon flow to AIR via de novo purine biosynthesis that causes the APB pathway to be required for prototrophic growth. Further work is under way to distinguish between this and other possible models.

ACKNOWLEDGMENTS

We thank George O'Toole for technical advice on the ApbA overexpression experiments.

This work was supported by N.I.H. grant GM47296 to D. M. Downs.

REFERENCES

1. **Benson, N. P., and B. S. Goldman.** 1992. Rapid mapping in *Salmonella typhimurium* with Mu*d*-P22 prophages. J. Bacteriol. **174:**1673–1681.
2. **Bochner, B. R., H. Huang, G. L. Schieven, and B. N. Ames.** 1980. Positive selection for loss of tetracycline resistance. J. Bacteriol. **143:**926–933.
3. **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.
4. **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.
5. **Chan, 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.
6. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
7. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis program for the VAX. Nucleic Acids Res. **12:**378–395.
8. **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.
9. **Downs, D. M., and J. R. Roth.** 1991. Synthesis of thiamine in *Salmonella typhimurium* independent of the *purF* function. J. Bacteriol. **173:**6597–6604.
10. **Escalante-Semerena, J. C., and J. R. Roth.** 1987. Regulation of cobalamin biosynthetic operons in *Salmonella typhimurium*. J. Bacteriol. **169:**2251–2258.
11. **Estramareix, B., D. Gaudry, and M. Therisod.** 1977. Biosynthesis of the thiamine thiazole in *Escherichia coli*. Biochimie (Paris) **59:**857–859.
12. **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.
13. **Goldstein, G. A., and G. M. Brown.** 1963. The biosynthesis of thiamine. V. Studies concerning precursors of the pyrimidine moiety. Arch. Biochem. Biophys. **103:**449–452.

13a. **Hollenback, J., and D. Downs.** Unpublished data.

14. **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.
15. **Hughes, K. T., and J. R. Roth.** 1985. Directed formation of deletions and duplications using Mu*d* (Ap, Lac). Genetics **109:** 263–282.
16. **Kilstrup, M., L. M. Meng, J. Neuhard, and P. Nygaard.** 1989. Genetic evidence for a repressor of synthesis of cytosine deaminase and purine biosynthesis enzymes in *Escherichia coli*. J. Bacteriol. **171:**2124–2127.
17. **Kleckner, J., J. R. Roth, and D. Botstein.** 1977. Genetic engineering *in vivo* using translocatable drug resistant elements. J. Mol. Biol. **116:**125–159.
18. **Maloy, S. R., and W. D. Nunn.** 1981. Selection for loss of tetracycline resistance by *Escherichia coli*. J. Bacteriol. **145:**1110–1112.
19. **Martinez, E., B. Bartolome, and F. de la Cruz.** 1988. pACYC184-derived cloning vectors containing the multiple cloning site and *lacAa* reporter gene of pUC8/9 and pUC18/19 plasmids. Gene **68:**159–162.
20. **Messenger, L. J., and H. Zalkin.** 1979. Glutamine phosphoribosyl pyrophosphate amidotransferase from *Escherichia coli*. J. Biol. Chem. **254:**3382–3392.
21. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Newell, P. C., and R. G. Tucker.** 1967. New pyrimidine pathway involved in the biosynthesis of the pyrimidine of thiamine. Nature (London) **215:**1384–1385.
23. **Newell, P. C., and R. G. Tucker.** 1968. Biosynthesis of the pyrimidine moiety of thiamine. Biochem. J. **106:**279–287.
24. **Newell, P. C., and R. G. Tucker.** 1968. Precursors of the pyrimidine moiety of thiamine. Biochem. J. **106:**271–277.
25. **Rolfes, R., and H. Zalkin.** 1990. Purification of the *Escherichia coli* purine regulon repressor and identification of corepressors. J. Bacteriol. **172:**5637–5642.
26. **Rolfes, R. J., and H. Zalkin.** 1988. *Escherichia coli* gene *purR* encoding a repressor protein for purine nucleotide synthesis. J. Biol. Chem. **263:**19653–19661.
27. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
28. **Schmieger, H.** 1972. Phage P22 mutants with increased or decreased transduction abilities. Mol. Gen. Genet. **119:**75–88.
29. **Smith, R. H., and A. B. McAllan.** 1970. Nucleic acid metabolism in the ruminant. 2. Formation of microbial nucleic acids in the rumen in relation to the digestion of food nitrogen, and the fate of dietary nucleic acids. Br. J. Nutr. **24:**545–556.
30. **Tabor, S.** 1990. Expression using the T7 RNA polymerase/promoter system, p. 16.2.1–16.2.11. *In* P. A. Ausube, R. Brent, and R. E. Kingston (ed.), Current protocols in molecular biology. Wiley, New York.

30a. **Tang, G. M., and J. R. Roth.** Personal communication.

31. **Tso, J. Y., M. A. Hermodson, and H. Zalkin.** 1982. Glutamine phosphoribosylpyrophosphate amidotransferase from cloned *Escherichia coli purF*. J. Biol. Chem. **257:**3532–3536.
32. **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.
33. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218:**97–106.
34. **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.
35. **White, R. H., and F. B. Rudolph.** 1979. Biosynthesis of the pyrimidine moiety of thiamine in *Escherichia coli*: incorporation of stable isotope-labeled glycines. Biochemistry **18:**2632–2636.
36. **White, R. H.** 1978. Stable isotope studies on the biosynthesis of the thiazole moiety of thiamine in *Escherichia coli*. Biochemistry **17:**3833–3840.
37. **White, R. H., and F. B. Rudolph.** 1978. The origin of the nitrogen atom in the thiazole ring of thiamine in *Escherichia coli*. Biochim. Biophys. Acta **542:**340–347.
38. **Youderian, P., P. Sugiono, K. L. Brewer, N. P. Higgins, and T. Elliot.** 1988. Packaging specific segments of the Salmonella chromosome with locked-in Mu*d*-P22 prophages. Genetics **118:**581–592.