JOURNAL OF BACTERIOLOGY, Oct. 1996, p. 5676–5682
0021-9193/96/$04.00+0

Vol. 178, No. 19

Mutations in *apbC* (*mrp*) Prevent Function of the Alternative Pyrimidine Biosynthetic Pathway in *Salmonella typhimurium*

LESLIE PETERSEN AND DIANA M. DOWNS*

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

Received 6 May 1996/Accepted 26 July 1996

The alternative pyrimidine biosynthetic (APB) pathway can synthesize the 4-amino-5-hydroxymethyl-2-methyl pyrimidine (HMP) moiety of thiamine in *Salmonella typhimurium* independently of de novo purine biosynthesis. When mutants defective in function of the APB pathway were isolated, the predominant class (40%) were defective in a single locus we have designated *apbC*. Mutations in *apbC* block function of the APB pathway since they prevent growth of a *purF* mutant in the absence of thiamine. Lesions in *apbC* also cause a thiamine auxotrophy in strains proficient in purine biosynthesis when fructose is provided as the sole carbon and energy source. Results presented here are consistent with ApbC being involved in the conversion of aminoimidazole ribonucleotide to HMP, and we suggest that ApbC performs a redundant step in thiamine synthesis. Sequence analysis demonstrated that *apbC* mutations were alleles of *mrp*, a locus previously reported in *Escherichia coli* as a *metG*-related protein. We propose that this locus in *S. typhimurium* be designated *apbC* to reflect its involvement in thiamine synthesis.

Our research seeks to understand how a cell coordinates its vast array of biochemical pathways to produce an efficient and adaptable metabolism. To be both efficient and flexible, the cell must maintain metabolic and regulatory cross talk between a number of pathways. We use the biosynthesis of thiamine, or vitamin B_1, in *Salmonella typhimurium* as a model system for studying pathway cross talk (24).

Thiamine is an essential vitamin for *S. typhimurium* and consists of a 4-amino-5-hydroxymethyl-2-methyl pyrimidine (HMP) moiety and a 4-methyl-5-(β-hydroxyethyl)thiazole moiety (THZ) (Fig. 1). Phosphorylated derivatives of HMP and THZ are synthesized by separate pathways before being joined to form thiamine phosphate. Thiamine phosphate is then phosphorylated by the *thiL* gene product to generate thiamine pyrophosphate (TPP), the biologically active form of thiamine (5, 17). Although metabolic precursors to both the HMP moiety and the THZ moiety are known, analysis of the biochemical steps involved in their synthesis is in its early stages (2, 30, 34). In the case of HMP, labeling studies have shown that the imidazole ring of aminoimidazole ribonucleotide (AIR) is cleaved between C-4 and C-5 and a two-carbon unit from ribose is inserted to form the pyrimidine ring (14, 15, 19, 35). Additional studies showed that all of the carbons in HMP are derived from AIR (16, 37).

Currently, our research seeks to identify and understand the pathways contributing to synthesis of the HMP moiety of thiamine. Synthesis of HMP can occur either through a well-studied pathway involving de novo purine biosynthesis or via the recently discovered alternative biosynthetic pyrimidine (APB) pathway (10). Studies by Newell and Tucker demonstrated that in *S. typhimurium* AIR is an intermediate in the formation of both purines and HMP (21, 22). Under the growth conditions used in their studies (aerobic growth on glucose), all mutants blocked prior to the formation of AIR required a source of purines and thiamine; this result was consistent with a common pathway branching to HMP or purines. It has recently become clear that growth of *S. typhimurium* in glucose medium prevented these and other investigators from realizing the potential contribution of the APB pathway to HMP biosynthesis (24). Evidence indicates that APB-dependent thiamine synthesis occurs in a *purF* genetic background on carbon sources that lead to the formation of ribose-5-phosphate (with the exception of glucose) (13, 24). Insertions in the *apbA* gene eliminate the functioning of the APB pathway (11). Work presented here and elsewhere has shown that lesions in a number of genetic loci eliminate the functioning of the APB pathway. In addition to ApbA, we have demonstrated a requirement for Gnd (gluconate-6-dehydrogenase), Zwf (glucose-6-dehydrogenase), and PurR (a transcriptional repressor) for the functioning of the APB pathway (11, 13, 24). Our current view of metabolic inputs into HMP biosynthesis is schematically illustrated in Fig. 1.

To identify structural genes in the APB pathway, additional mutants preventing the functioning of this pathway were isolated. The predominant class of mutants were defective in a single genetic locus we have designated *apbC*. We report here the initial genetic and physical characterization of *apbC* mutants, and we demonstrate that *apbC* mutations are lesions in *mrp*, an open reading frame identified in *Escherichia coli* as a *metG*-related protein (8). Results presented here are consistent with the model that ApbC is needed for the conversion of AIR to HMP. We recommend that, in *S. typhimurium*, *mrp* be designated *apbC* to reflect the role this locus has in thiamine biosynthesis.

MATERIALS AND METHODS

Bacterial strains. All strains in this study are derivatives of *S. typhimurium* and are listed with their genotypes in Table 1. Tn*10d*(Tc) refers to the transposition-defective mini-Tn*10* described by Way et al. (33). MudJ is used throughout the article to refer to the Mud dl1734 transposon, which has been described previously (6).

Culture media and chemicals. No-carbon E medium supplemented with $MgSO_4$ (1 mM) (9, 31) and a carbon source (11 mM) was used as minimal medium. Difco nutrient broth (8 g/liter) with NaCl (5 g/liter) was used as rich medium. Difco Bitek agar was added (15 g/liter) for solid medium. Unless otherwise noted, adenine and thiamine were added to final concentrations of 0.4 mM and 100 nM, respectively. The final concentrations of the antibiotics in rich medium were as follows (in micrograms per milliliter): ampicillin, 30; chloramphenicol, 20; kanamycin, 50; tetracycline, 20. In minimal medium the final con-

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

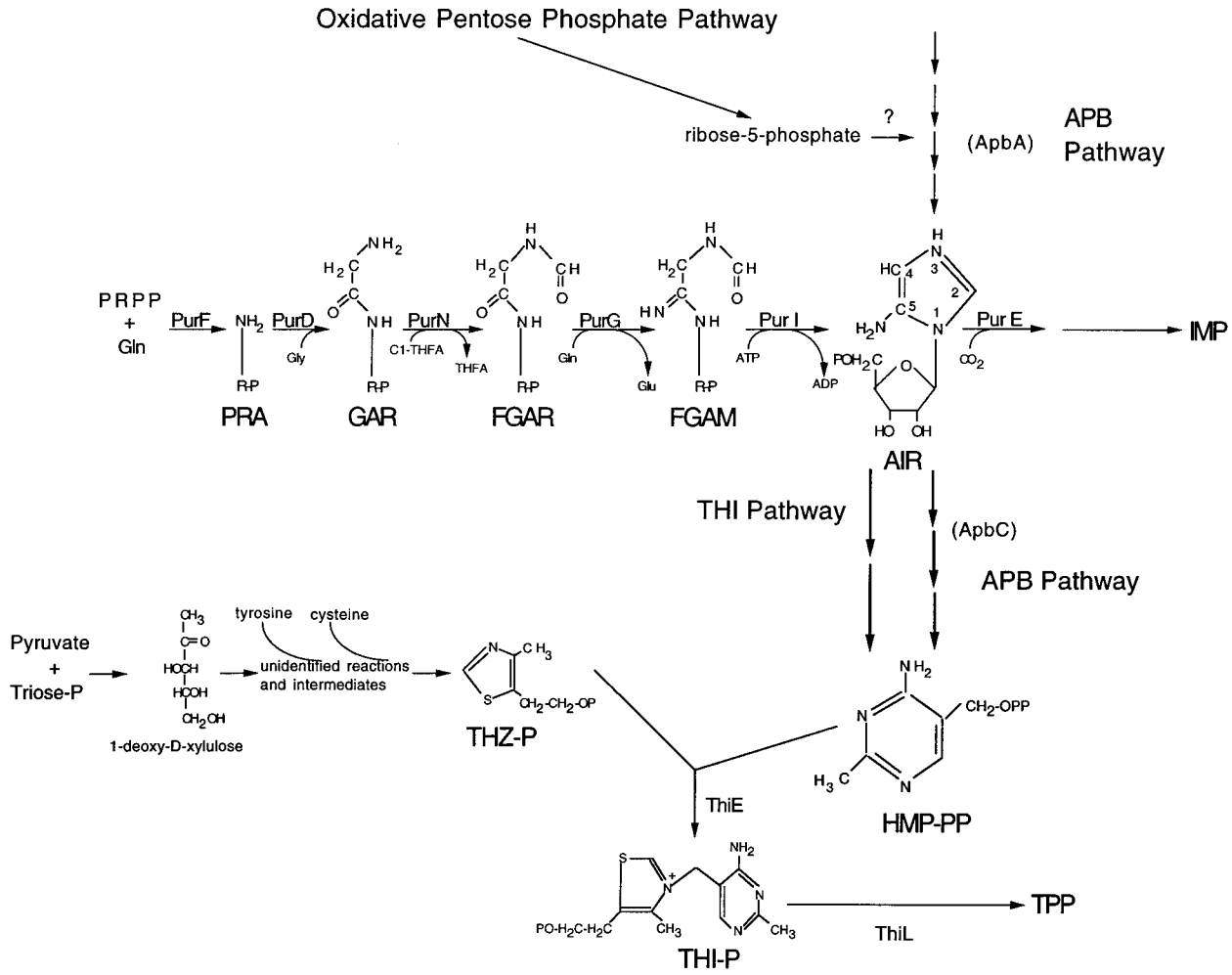

FIG. 1. Metabolic inputs and a model for the role of ApbC in the synthesis of HMP. De novo synthesis of purines and HMP is schematically illustrated. The gene products required for selected reactions are indicated above the relevant arrows. The number of steps involved in the formation of HMP from AIR is not known, nor is the number of steps in the APB pathway leading to the formation of AIR. Ribose-5-phosphate is a postulated intermediate in the APB pathway (13). Two pathways from AIR to HMP are proposed: (i) the THI pathway, requiring high AIR concentrations, and (ii) the APB pathway, functional with low AIR concentrations. The THI pathway is defined by conditions that allow thiamine synthesis independent of ApbC. Abbreviations: PRA, phosphoribosyl amine; AIR, aminoimidazole ribonucleotide; THZ-P, 4-methyl-5-(β-hydroxyethyl)thiazole phosphate; HMP-PP, 4-amino-5-hydroxymethyl-2-methyl pyrimidine pyrophosphate; GAR, glycinamide ribonucleotide; FGAR, formylglycinamide ribonucleotide; FGAM, formyl glycinamidine ribonucleotide; TPP, thiamine pyrophosphate.

centrations of these antibiotics were 15, 4, 125, and 10 μg/ml, respectively. All chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Transduction methods. Transductions were performed with the high-frequency, generalized-transduction bacteriophage P22 mutant HT105/1 *int-201* (29) as described previously (11). Transductants were purified and identified as phage free by streaking and cross-streaking on green plates (7).

Mutant isolation. (i) Apb mutants. An Apb$^-$ phenotype has been defined by two criteria: (i) the inability to synthesize thiamine in a *purF* genetic background when grown on minimal medium containing adenine with gluconate, glucose (with trace minerals), or glycerol as the sole carbon source and (ii) the ability to synthesize thiamine in a *pur*$^+$ genetic background on minimal glucose medium (24). Mutants with an Apb$^-$ phenotype were isolated by insertion mutagenesis using one of two defective transposons [Tn*10d*(Tc) or MudJ]. In each case, a P22 lysate was grown on a pool of cells containing >70,000 clones, where each clone contained one of the respective elements inserted randomly into the chromosome (18).

Recipient cells of *purF2085* (DM1936) were transduced to the appropriate antibiotic resistance on nutrient agar plates. The antibiotic-resistant transductants were replica plated sequentially to (i) a minimal-antibiotic plate supplemented with adenine and (ii) a minimal-antibiotic plate supplemented with adenine and thiamine. Putative Apb$^-$ mutants were identified by their inability to grow without exogenous thiamine. After being streaked out and verified as phage sensitive, these mutants were reconstructed by transducing the insertion into both a *purF2085* (DM1936) background and a wild-type (LT2) background. Only insertions 100% linked to the mutant phenotype in *purF* and allowing growth on minimal glucose medium in a wild-type (*pur*$^+$) genetic background were pursued, thus eliminating mutations in de novo *pur* or *thi* genes. The 14 mutants described here were a result of independent mutant searches using fructose, glycerol, and gluconate as sole carbon sources. A typical search involved screening 20,000 antibiotic-resistant colonies and resulted in two or three mutants with an Apb$^-$ phenotype.

(ii) Mutants that could utilize AIR_s. It had been reported that the thiamine requirement of *pur* mutants could be satisfied by exogenous aminoamidazole riboside (AIR_s). Newell and Tucker demonstrated that a spontaneous mutation increased the permeability of strains to AIR_s (21). To facilitate nutritional analysis, we identified a spontaneous mutant derivative of *purF2085* (DM1936) whose thiamine requirement was satisfied when 3 nmol of AIR_s (in 1 μl) was spotted on the surface of a plate (DM2253). Under the same plating conditions, the parent strain (DM1936) required 284 nM AIR_s to produce similar growth in the absence of thiamine, 94 times that for DM2253 (*purF2085* AIR_s).

Derivatives of *purF2085* with an increased ability to take up AIR_s were obtained as follows. (i) Strain DM1936 (*purF2085*) was plated in top-agar overlays on a minimal glucose adenine plate, and 142 nmol of AIR_s (in 1 μl) was spotted on the surface. (ii) Spontaneous revertants arising around the AIR_s were tested for their ability to grow without thiamine in the presence of AIR_s. One such mutant was saved and used for subsequent strain constructions (DM2253). AIR_s was kindly provided by J. Stubbe, Massachusetts Institute of Technology.

Phenotypic characterization. Mutant phenotypes were assessed by liquid growth curves and on solid growth media.

(i) Growth curves. Strains to be analyzed were grown to full density in nutrient

TABLE 1. Strains

Strain	Genotype
LT2	Wild type
DM176	*thi-938*::MudJ[a]
DM270	*apbC55*::Tn*10d*(Tc)[b]
DM299	*apbC56*::Tn*10d*(Tc)
DM757	*purF2085 apbC53*::Tn*10d*(Tc)
DM760	*purF2085 apbC54*::Tn*10d*(Tc)
DM809	*purF2085 apbC51*::Tn*10d*(Tc)
DM810	*purF2085 apbC52*::Tn*10d*(Tc)
DM839	*apbC57*::Tn*10d*(Tc)
DM914	*purF2085 zed-8045*::Tn*10d*(Cm)
DM930	*purF2085 apbC55*::Tn*10d*(Tc)
DM983	*purF2085 apbC83*::Tn*10d*(Tc)
DM1001	*purF2085 apbC58*::Tn*10d*(Tc)
DM1002	*purF2085 apbC61*::Tn*10d*(Tc)
DM1003	*purF2085 apbC62*::Tn*10d*(Tc)
DM1004	*purF2085 apbC63*::Tn*10d*(Tc)
DM1005	*purF2085 apbC60*::Tn*10d*(Tc)
DM1006	*purF2085 apbC59*::Tn*10d*(Tc)
DM1098	*purF2085 thi-940*::MudQ
DM1100	*purF2085 purE2154*::MudJ *apbC55*::Tn*10d*(Tc)
DM1108	*thi-939*::MudP
DM1391	*apbC55*::Tn*10d*(Tc)/pApbC-1
DM1440	*purF2085 apbC55*::Tn*10d*(Tc)/pApbC-1
DM1936	*purF2085*
DM2134	*apbC57*::Tn*10d*(Tc) *thi-939*::MudP
DM2135	*apbC55*::Tn*10d*(Tc) *thi-939*::MudP
DM2156	*apbC53*::Tn*10d*(Tc) *thi-939*::MudP
DM2252	*purF2085 zee-78*::Tn*10 metG319*
DM2253	*purF2085* AIR_s
DM2254	*purF2085* AIR_s *apbC53*::Tn*10d*(Tc)
DM2324	*cra-51*::Tn*10d*(Tc) *apbC67*::MudJ
TT2242	*aroD5 hisW1824 purF145 rpsL metG319 zee-78*::Tn*10*
SA2026	*metG419*

[a] MudJ is used throughout the text to refer to the Mud dl1734 transposon (6).
[b] Tn*10d*(Tc) refers to the transposition-defective mini-Tn*10* (Tn*10*Δ-*16*Δ-*17*) (33).

medium at 37°C. After overnight incubation, cells were pelleted and resuspended in an equal volume of sterile NaCl (145 mM). A 1/33 (vol/vol) inoculum was made into 5 ml of the appropriate medium. Culture tubes were placed in a shaking water bath at 37°C, and growth was monitored by optical density at 650 nm on a Bausch & Lomb Spectronic 20. Specific growth rate was determined by using $\mu = \ln(X/X_0)/T$, where X is A_{650} during the linear portion of the growth curve and T is time. Routinely, the A_{650} at time zero was between 0.02 and 0.04. Growth of overnight cultures was scored as negative if the A_{650} was <0.3 when the control cultures with added thiamine reached an A_{650} of >1.0. When needed, trace minerals were added at concentrations specified elsewhere (3).

(ii) Solid growth media tests. Nutritional requirements were measured in soft-agar overlays. A 0.2-ml aliquot of a saline cell suspension was added to 3 ml of molten 0.7% agar and spread on the appropriate solid medium. After the agar solidified, nutrients to be tested were spotted as follows: 1 μl of 100 μM thiamine and 10 μl of 100 μM pantothenate. The plates were then incubated at 37°C overnight before growth was scored.

Molecular-biological techniques. Standard methods were used for DNA restriction endonuclease digestion and ligation. Restriction enzymes and ligase were purchased from Promega (Madison, Wis.). Plasmid DNA for sequencing was isolated with the Wizard Mini-prep kit purchased from Promega and was transferred between strains by electroporation using a Bio-Rad *E. coli* Pulser (Bio-Rad Laboratories, Richmond, Calif.).

(i) Cloning *apbC*. Allele *thi-938*::MudJ [55% cotransducible with *apbC55*::Tn*10d*(Tc)] was converted to its MudP and MudQ derivatives as described elsewhere (4). The Mud element in each of the resulting strains was induced to generate two lysates containing DNA from either side of the Mud insertion. The DNA from each induction was purified from phage heads, digested with *Hin*dIII and ligated into the multiple cloning site of pSU19 as previously described (11). The two ligation mixes were separately electroporated into DM270 [*apbC55*::Tn*10d*(Tc)], and complementation of the *apbC* phenotype was scored. Only DNA packaged from the induction of *thi-939*::MudP (DM1108) yielded a complementing clone. This clone contained a 4.0-kb insert and was designated pApbC-1.

(ii) DNA sequencing. Plasmid DNA sequences were determined from denatured double-stranded templates by the dideoxynucleotide chain termination method (28) 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.). The −40 and reverse primers for M13mp19 were purchased from New England Biolabs. Additionally, the sequence of a primer to the left end of the Tn*10d*(Tc)-defective transposon was determined (5′ TCCA TTGCTGTTGACAAAGG 3′), and the primer was generated by Fisher Oligo-to-Go (Chicago, Ill.).

Chromosomal DNA was sequenced with the Sequitherm cycle sequencing kit from the Epicentre Technologies Corporation (Madison, Wis.) and [^{32}P]ATP with a specific activity of >6,000 Ci/mmol (Dupont). A preparation of DNA enriched for the region carrying the desired *apbC*::Tn*10d*(Tc) was obtained by inducing the *thi-939*::MudP locked-in prophage (4) in the double-mutant strain [*thi-939*::MudP *apbC*::Tn*10d*(Tc)] by the method of Youderian et al. (38).

PCR amplification. A Thermolyne Temp-Tronic thermocycler was used to amplify DNA between *metG* and various *apbC*::Tn*10d*(Tc) insertions. Primers designed for amplification were 5′ GAACAGGTTGTTCGGCTTA 3′, internal to *metG*, and 5′ GACAAGATGTGTATCCACCTTAAC 3′, insertion sequences of Tn*10d*(Tc) (33). In vitro amplification was performed as previously described (34). Vent (exo-) was purchased from New England Biolabs. The size of the amplified fragment was determined by agarose gel electrophoresis and comparison with known size standards.

Computer analysis. BLAST (Basic Local Alignment Search Tool) was used to search for homologous sequences in the GenBank and SwissProt databases as data were generated (1).

RESULTS

The ability of *purF* mutants to grow in the absence of exogenous thiamine on minimal adenine medium with gluconate, glucose (with trace minerals), or glycerol as the sole carbon source has been attributed to the APB pathway (24).

Isolation of *apbC* mutants. In an attempt to identify enzymatic steps in the APB pathway, insertion mutagenesis was used to isolate mutants of *purF2085* (DM1936) defective in APB pathway function. A P22 lysate grown on a pool of cells containing >70,000 insertions [Tn*10d*(Tc) or MudJ] was used to transduce *purF2085* to tetracycline or kanamycin resistance. Approximately 150,000 antibiotic-resistant transductants were screened, resulting in 33 mutants with an Apb$^-$ phenotype. Of these mutants, 4 carried MudJ insertions and 29 carried Tn*10d*(Tc) insertions; 4 of the 33 mutants carried lesions in *gnd* (13, 24). To identify linkage groups within the remaining 29 mutants, a Tn*10d*(Cm) element [*zed-8045*::Tn*10d*(Cm)] 88% linked to the *apb* lesion in DM270 [*apbC55*::Tn*10d*(Tc)] was isolated (12, 18). Transductional linkage analysis demonstrated that 15 of the mutants were defective in a locus closely linked (80 to 95%) to *zed-8045*::Tn*10d*(Cm). Subsequent work determined that 1 of the linked mutations (*thi-938*::MudJ) resulted in a leaky Thi$^-$ phenotype and was defective in a locus distinct from the remaining 14 Tn*10d*(Tc) insertions mutations. The single locus affected by these 14 mutations is referred to as *apbC* throughout the article.

ApbC insertions map to the *mrp* gene. The *apbC* locus was cloned by using MudP22 technology to enrich for neighboring *apbC*$^+$ DNA (38). A representative plasmid that complemented DM270 [*apbC55*::Tn*10d*(Tc)] was designated pApbC-1 and found to contain a 4.0-kb insert. Sequencing of the pApbC insert using the −40 and reverse primers for M13mp19 identified homology to *yehC* and *metG* of *Escherichia coli*, respectively (Fig. 2). *metG* encodes the methionyl tRNA synthetase gene (8), and *yehC* encodes a 282-nucleotide open reading frame according to sequence data from systematic sequencing around min 46.6 of the *E. coli* genome (32). On the basis of this sequence analysis three additional open reading frames were predicted to be included on pApbC-1: *yehD*, *yehE*, and *mrp* (32).

To assign *apbC* lesions to one of the predicted open reading frames on pApbC-1, the DNA sequences adjacent to three *apbC*::Tn*10d*(Tc) insertions were determined. The sequences obtained were analyzed by BLAST using the SwissProt database. In each case the predicted amino acid sequence was

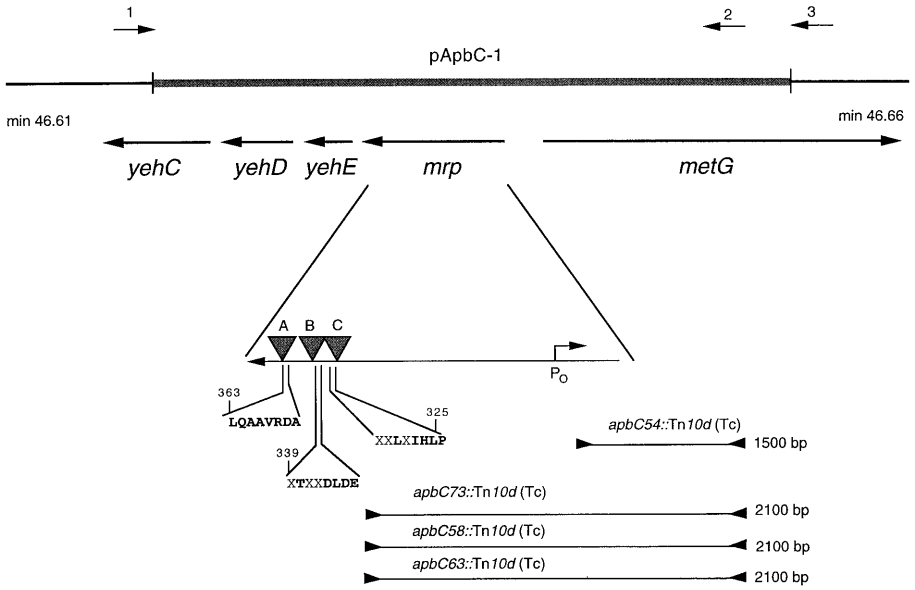

FIG. 2. Mutations in *apbC* are alleles of *mrp*. The region between min 46.61 and 46.66 of the *E. coli* chromosome is shown. Arrows 1, 2, and 3 refer to the −40, *metG*, and reverse primers, respectively (see Materials and Methods). *yehD*, *yehE*, and *yehC* are open reading frames of unknown function identified as part of the *E. coli* genome sequencing project. The locations of three insertions in the *mrp* gene are shown [A, *apbC57*::Tn*10d*(Tc); B, *apbC53*::Tn*10d*(Tc); C, *apbC55*::Tn*10d*(Tc)], as determined by sequencing directly out of the respective Tn*10d*(Tc); numbers refer to amino acids from the *E. coli mrp* open reading frame. The amino acid residues conserved between the *E. coli* and *S. typhimurium* predicted sequences are boldfaced. P_0, promoter internal to *mrp* that can be used to transcribe *metG* (8). Four additional *apbC* mutations mapped by in vitro amplification are diagrammed below.

homologous with a portion of the *E. coli* Mrp protein (1). The results (Fig. 2) mapped three *apbC* insertions within the *mrp* open reading frame of *E. coli* and were consistent with P22 transductions showing that *apbC*::Tn*10d*(Tc) was >90% linked to two independent *metG* point mutants (SA2026 and TT2242). Each of the three insertions we sequenced eliminated fewer than 55 amino acids at the C-terminal end of the predicted 379-amino-acid protein.

PCR mapping of remaining *apbC* insertions. Previous work with *E. coli* had demonstrated the existence of a promoter internal to *mrp* that could direct transcription of *metG* both in vivo and in vitro (8). None of the three *apbC* insertions we mapped would disrupt transcription from this promoter (Fig. 2). To address the possible role for this promoter, we mapped 4 of the remaining 11 mutations using in vitro DNA amplification. Primers to the sequence internal to *metG* and the insertion sequence of the Tn*10d*(Tc) were used for amplification. The lengths of the amplification products identified the approximate locations of the insertions within the *apbC* sequence (Fig. 2). Three of the insertions yielded amplification products of approximately 2,100 bp [*apbC73*::Tn*10d*(Tc), *apbC58*::Tn*10d*(Tc), and *apbC60*::Tn*10d*(Tc)]; one [*apbC54*::Tn*10d*(Tc)] yielded a 1,500-bp product. The phenotype of a strain carrying the latter insertion [DM761, *apbC54*::Tn*10d*(Tc)] was indistinguishable from those with other *apbC* alleles. Since we mapped seven insertions to the *apbC* gene, we suggested that polarity on downstream genes was not responsible for the phenotypes we observed.

Mutations in *apbC* prevent APB-dependent thiamine synthesis. Because our understanding of the APB pathway is in its early stages, we sought to correlate the phenotypes of *apbC* mutants with those of other mutants identified as defective in the APB pathway. Like all Apb$^-$ mutants isolated so far (24), *apbC* mutations eliminated the growth of a *purF2085* deletion strain on minimal adenine medium when gluconate, glycerol, or glucose (with trace minerals) was used as a carbon source. Data in Table 2 illustrate this phenotype on different carbon sources. The APB pathway functions in a *purF* strain under all conditions shown; it does not function in *purF apbC* mutants, although growth is restored by the addition of exogenous thiamine. On the basis of these phenotypes, we have defined *apbC* as a genetic locus required for APB-dependent thiamine synthesis.

Further characterization of *apbC* mutants uncovered pheno-

TABLE 2. Growth rates of *purF2085* and *purF2085 apbC55*::Tn*10d*(Tc) strains on various carbon sources[a]

Strain	Specific growth rate (μ)[b]							
	Gluc + Ade		Gly + Ade		Glcn + Ade		Rib + Ade	
	−Thi	+Thi	−Thi	+Thi	−Thi	+Thi	−Thi	+Thi
DM1936 *purF2085* (Δ)	0.53	0.68	0.43	0.59	0.48	0.60	0.43	0.37
DM930 *purF2085 apbC55*::Tn*10d*	0.01	0.78	0.07	0.58	0.11	0.70	0.29	0.37
DM1100 *purF2085 apbC55*::Tn*10d purE2154*::MudJ	0.60	0.68	0.41	0.54	0.40	0.48	ND	ND

[a] Liquid growth analysis was performed and growth rates were determined as described in the Materials and Methods. Trace minerals were added to the media at concentrations specified elsewhere (3).

[b] Data from representative experiments. Abbreviations: Ade, adenine; Thi, thiamine; Glcn, gluconate; Glu, glucose; Gly, glycerol; Rib, ribose; ND, not determined.

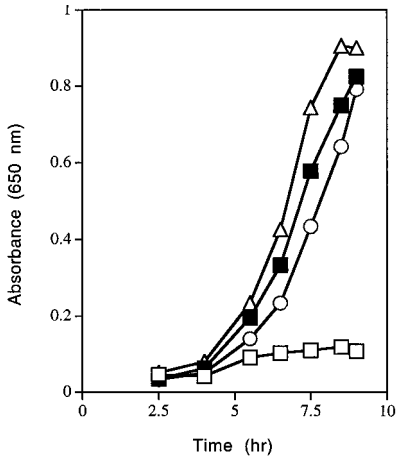

FIG. 3. Mutations in the *apbC* gene cause a conditional thiamine auxotrophy. Cultures were grown at 37°C in shaking water baths as described in Materials and Methods (except that the cells were washed once in saline) Open squares and triangles, growth of *apbC55*::Tn*10d*(Tc) (DM270) and LT2, respectively, in minimal fructose medium; circles, growth of DM270 in minimal gluconate medium; filled squares, growth in medium with adenine and thiamine.

types distinct from those of previously identified *apbA* mutants. Unlike *apbA* lesions, mutations in *apbC* did not eliminate growth of *purF2085* on adenine when ribose was used as a sole carbon source (Table 2). In contrast to strain DM587 [*purF2085 apbA7*::Tn*10d*(Tc)], which requires pantothenate or thiamine (13), pantothenate was unable to satisfy the thiamine requirement of strain DM930 [*purF2085 apbC55*::Tn*10d*(Tc)]. The reason for these phenotypic differences is currently unknown.

The results presented in the following sections are consistent with the model that AIR is converted to HMP by alternate (or partially duplicated) pathways. One of these pathways (the APB pathway) is dependent on ApbC. The second pathway (THI) is independent of ApbC, requires elevated levels of AIR, and is inhibited by catabolism of fructose as the sole carbon source.

***apbC* mutants are defective in the conversion of AIR to HMP.** We had previously shown that a mutation in *purE* restored the functioning of the APB pathway in a *purF* strain on adenine glucose medium by preventing diversion of AIR to IMP synthesis (24). As expected, a mutation in *apbA* eliminated thiamine synthesis in a *purE purF* double mutant (Fig. 1) (24). Results in Table 2 show that blocking *purE* allowed thiamine synthesis in *purF apbC* mutants. A *purE* mutation restored thiamine synthesis in strain DM930 (*purF apbC*) on minimal adenine medium with glucose (and trace minerals), glycerol, or gluconate. These results were consistent with the view that *apbC* mutants were not blocked in the formation of AIR.

ApbC is required for thiamine synthesis on minimal fructose medium. The phenotype of an *apbC* mutant in a wild-type (Pur^+) background was examined. The growth rates of strain DM270 [*apbC55*::Tn*10d*(Tc)] and a wild-type strain (LT2) were comparable on minimal medium with glycerol, glucose, or gluconate as the sole carbon source. This was the expected phenotype for a mutant with a mutation in the APB pathway (10, 24).

However, *apbC* mutants were unable to grow in the absence of thiamine when fructose was used as the sole carbon source. This conditional thiamine requirement of *apbC* mutants is shown in Fig. 3. The ability of our assay (ApbC-dependent thiamine synthesis) to distinguish between catabolism of glucose and catabolism of fructose was unexpected. The biochemical literature predicted that the carbon flux and metabolites produced in each case would be similar (20). However, fructose catabolism might be unique in affecting the global regulator Cra (formerly FruR) by providing the effectors fructose-1-phosphate and fructose-1,6-bisphosphate (25–27).

To address an involvement of Cra in the inhibition of thiamine synthesis, we constructed a *cra apbC* double mutant. The thiamine requirements of the *apbC* (DM2255) and *apbC cra* (DM2324) strains were indistinguishable when either gluconate or fructose was provided as a sole carbon source (data not shown). In addition, an effect mediated through Cra might predict that the presence of fructose in addition to a second carbon source (e.g., gluconate) would prevent thiamine synthesis in an *apbC* mutant. The growth of strain DM270 [*apbC55*::Tn*10d*(Tc)] on gluconate was indistinguishable from that on fructose gluconate (final A_{650}s, 1.19 and 1.38, respectively), indicating that fructose prevented thiamine synthesis only when it was the sole carbon source.

The conditional thiamine auxotrophy of *apbC* mutants on fructose, and the stimulatory effect of a *purE* mutation described above, supported two pathways for the conversion of AIR to HMP, one dependent on and one independent of ApbC.

Increased levels of AIR_s eliminate the Apb^- phenotype of *apbC* mutants. To address the prediction of our model that the THI pathway required increased AIR concentrations, we titrated the levels of exogenous AIR_s needed to satisfy the thiamine requirement of *apbC* mutants. Various cellular kinases are thought to phosphorylate AIR_s, thus allowing it to enter cellular pools of purine intermediates as AIR (21). Since ribosylated intermediates are not taken up efficiently, we isolated a derivative of *purF2085* able to utilize AIR_s to satisfy the thiamine requirement of the strain (see Materials and Methods). This mutation is presumed to affect the cell envelope and facilitate uptake of ribosylated intermediates (23, 36). Similar mutants were used to demonstrate that AIR was a common intermediate between the thiamine and purine biosynthetic pathways (21).

To compare the AIR requirements of *purF2085* and *purF2085 apbC55*::Tn*10d*(Tc), we provided AIR_s to the appropriate strains in top-agar overlays on minimal adenine medium with glucose as a carbon source. This allowed us to assess the AIR requirement of strains unable to use the APB pathway for the conversion of AIR to HMP (24). In agreement with the model presented, we found that strain DM2254 [*purF2085* AIR_s *apbC53*::Tn*10d*(Tc)] required approximately twofold more exogenous AIR_s to satisfy its thiamine requirement than the parent strain DM2253 (*purF2085* AIR_s) (7 versus 3 nmol in 1 μl, respectively). While not definitive, these data were consistent with the above results suggesting that increased AIR concentration allows the THI pathway to function independently of ApbC in HMP biosynthesis.

DISCUSSION

The work presented here represents the initial genetic and physical characterization of a new locus required for APB-dependent thiamine synthesis in *S. typhimurium*. Mutations characterized herein define the *apbC* locus, previously identified as *mrp* in *E. coli*. Mutations in *apbC* prevented aerobic growth of a *purF2085* deletion strain on medium containing adenine in all conditions shown to allow thiamine synthesis via the APB pathway. In each case growth was restored by the addition of thiamine. To reflect its role in thiamine synthesis,

we propose that the *mrp* homolog in *S. typhimurium* be designated *apbC*.

Analysis of the *mrp* sequence was previously performed by Dardel et al., and the predicted protein was found to have a consensus nucleotide binding site [GXXXXGK(ST)] found in many ATPases and GTPases (8). This binding site motif suggests that *apbC* may encode a protein with ATP hydrolyzing activity, possibly associated with an enzymatic step in the APB pathway.

Previous work has identified AIR as a central metabolite in thiamine synthesis whose levels are determined by several enzymes including PurF and its alternate (13), PurGNDI (21), PurE and (indirectly) PurR (24), and ApbA (11). The analysis of *apbC* mutants has significantly advanced our understanding of thiamine synthesis in *S. typhimurium*, and the data presented herein support the model that AIR is converted to HMP by alternate (or partially duplicated) steps. These include (i) an ApbC-dependent route, thought to be an extension of the APB pathway, and (ii) an ApbC-independent route, the THI pathway. The THI pathway for conversion of AIR to HMP requires higher levels of AIR than does the APB pathway. We can envision several mechanisms that would allow for differential AIR utilization. One attractive possibility is that the AIR-utilizing enzymes in the APB and THI pathways have a low K_m and a high K_m for AIR, respectively. This hypothesis can be addressed once the appropriate enzymes have been identified.

Of the two routes to HMP, only the APB pathway is functional when fructose is the sole carbon source since ApbC is required for thiamine-independent growth in this medium. The distinctive effect of fructose suggested an involvement of the global regulator Cra. Fructose catabolism would provide the effectors for this regulator, fructose-1-phosphate and fructose-1,6-biphosphate (25, 26). Cra has been shown to be involved in the regulation of a number of genes in carbon metabolism and is thought to modulate direction of carbon flow (25). A direct involvement of Cra was not supported by our results: (i) a *cra apbC* double mutant was not auxotrophic for thiamine on carbon sources other than fructose, and (ii) an *apbC* mutant was prototrophic when fructose was present but not as the sole carbon source. Additional work is necessary to determine the role of fructose catabolism in inhibiting thiamine synthesis.

In summary, the results presented here are consistent with a model involving two routes for the conversion of AIR to HMP (Fig. 1). We suggest that these two pathways are utilized by the cell under different metabolic conditions. Our model specifies that ApbC is a component of the APB pathway, which can utilize low concentrations of AIR possibly generated via the upper branch of the APB pathway. We suggest that ApbC-independent thiamine synthesis is due to the THI pathway, which requires elevated levels of AIR to function, and is inhibited when fructose is used as a sole carbon source.

While we refer to them as pathways, it is important to emphasize that the routes of HMP synthesis may involve "side reactions" of enzymes with established functions. It is possible that enzymes with relatively broad specificities could utilize alternate substrates to produce products that can be used in low-flux pathways such as thiamine synthesis. Alternatively, as we have shown in one case, metabolites found in other pathways may be utilized to satisfy a low (i.e., vitamin) requirement (13). Regardless, the existence of such metabolite or by-product scavenging fits our definition as a form of metabolic cross talk and reflects a strategy the cell can use to achieve the metabolic flexibility necessary to remain competitive in a changing environment.

The differential utilization of the THI and APB pathways may reflect regulation the cell uses to ensure thiamine synthesis independent of flux through the purine pathway. This strategy could be advantageous since flux through the purine pathway would change depending on the levels of exogenous purines available in the environment. By understanding the mechanism(s) the cell uses to differentially utilize these pathways; we will further understand how cellular metabolism can be regulated to optimize growth under different conditions.

ACKNOWLEDGMENTS

We thank J. Stubbe for supplying AIR_s and Todd Christian for the initial sequencing of pApbC-1.

This work was supported by NIH grant GM47296 to D. M. Downs.

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. **Balch, W. E., and R. S. Wolfe.** 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. Appl. Environ. Microbiol. **32:**781–791.
4. **Benson, N. P., and B. S. Goldman.** 1992. Rapid mapping in *Salmonella typhimurium* with Mu*d*-P22 prophages. J. Bacteriol. **174:**1673–1681.
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. **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.
8. **Dardel, F., M. Panvert, and G. Fayat.** 1990. Transcription and regulation of expression of the *Escherichia coli* methionyl-tRNA synthetase gene. Mol. Gen. Genet. **223:**121–133.
9. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
10. **Downs, D. M.** 1992. Evidence for a new, oxygen-regulated biosynthetic pathway for the pyrimidine moiety of thiamine in *Salmonella typhimurium*. J. Bacteriol. **174:**1515–1521.
11. **Downs, D. M., and L. Petersen.** 1994. *apbA*, a new genetic locus involved in thiamine biosynthesis in *Salmonella typhimurium*. J. Bacteriol. **176:**4858–4864.
12. **Elliot, T., and J. R. Roth.** 1988. Characterization of Tn*10d*-Cam: a transposition-defective Tn*10* specifying chloramphenicol resistance. Mol. Gen. Genet. **213:**332–338.
13. **Enos-Berlage, J. L., and D. M. Downs.** 1996. Involvement of the oxidative pentose phosphate pathway in thiamine biosynthesis in *Salmonella typhimurium*. J. Bacteriol. **178:**1476–1479.
14. **Estramareix, B.** 1970. Biosynthesis of the pyrimidine moiety of thiamine: origin of carbon-6 in *Salmonella typhimurium*. Biochim. Biophys. Acta **208:** 170–171.
15. **Estramareix, B., and M. Lesieur.** 1969. Biosynthesis of the pyrimidine moiety of thiamine: origin of carbons in positions 2 and 4 in *Salmonella typhimurium*. Biochim. Biophys. Acta **192:**375–377.
16. **Estramareix, B., and M. Therisod.** 1984. Biosynthesis of thiamine: 5-aminoimidazole ribotide as the precursor of all the carbon atoms of the pyrimidine moiety. J. Am. Chem. Soc. **106:**3857–3860.
17. **Imamura, N., and H. Nakayama.** 1982. *thiK* and *thiL* loci of *Escherichia coli*. J. Bacteriol. **151:**708–717.
18. **Kleckner, N., J. Bender, and S. Gottesman.** 1991. Uses of transposons with emphasis on Tn*10*. Methods Enzymol. **204:**139–180.
19. **Kumaoka, H., and G. Brown.** 1967. Biosynthesis of thiamine: incorporation of formate into carbon atom two of the pyrimidine moiety of thiamine. Arch. Biochem. Biophys. **122:**378–384.
20. **Lin, E. C.** 1987. Dissimilatory pathways for sugars, polyols, and carboxylates, p. 244–285. *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 of Microbiology, Washington, D.C.
21. **Newell, P. C., and R. G. Tucker.** 1968. Biosynthesis of the pyrimidine moiety of thiamine. Biochem. J. **106:**279–287.
22. **Newell, P. C., and R. G. Tucker.** 1968. Precursors of the pyrimidine moiety

of thiamine. Biochem. J. **106:**271–277.
23. **O'Toole, G. A., M. R. Rondon, and J. C. Escalante-Semerena.** 1993. Analysis of mutants of *Salmonella typhimurium* defective in the synthesis of the nucleotide loop. J. Bacteriol. **175:**3317–3326.
24. **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.
25. **Ramseier, T. M., S. Bledig, V. Michotey, R. Feghali, and J. Saier.** 1995. The global regulatory protein FruR modulates the direction of carbon flow in *Escherichia coli*. Mol. Microbiol. **16:**1157–1169.
26. **Ramseier, T. M., D. Negre, J. C. Cortay, M. Scarabel, A. J. Cozzone, and J. Saier.** 1993. *In vitro* binding of the pleiotropic transcriptional regulatory protein, FruR, to the *fru*, *pps*, *ace*, *pts*, *icd* operons of *Escherichia coli* and *Salmonella typhimurium*. J. Mol. Biol. **234:**28–44.
27. **Saier, M. H., Jr., and T. M. Ramseier.** 1996. The catabolite repressor/activator (Cra) protein of enteric bacteria. J. Bacteriol. **178:**3411–3417.
28. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
29. **Schmieger, H.** 1972. Phage P22 mutants with increased or decreased transduction abilities. Mol. Gen. Genet. **119:**75–88.
30. **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.
31. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218:**97–106.
32. **Wahl, R., P. Rice, C. M. Rice, and M. Kroger.** 1994. ECD—a totally integrated database of *Escherichia coli* K12. Nucleic Acids Res. **22:**3450–3455.
33. **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.
34. **Webb, E., F. Febres, and D. M. Downs.** 1996. Thiamine pyrophosphate (TPP) negatively regulates transcription of some *thi* genes of *Salmonella typhimurium*. J. Bacteriol. **178:**2533–2538.
35. **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. **Xu, K., J. Delling, and T. Elliott.** 1992. The genes required for heme synthesis in *Salmonella typhimurium* include those encoding alternative functions for aerobic and anaerobic coproporphyrinogen oxidation. J. Bacteriol. **174:**3953–3963.
37. **Yamada, K., and H. Kumaoka.** 1982. Biosynthesis of thiamin. Incorporation of a two-carbon fragment derived from ribose of 5-aminoimidazole ribotide into the pyrimidine moiety of thiamin. Biochem. Int. **5:**771–776.
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.