A Novel Involvement of the PurG and PurI Proteins in Thiamine Synthesis Via the Alternative Pyrimidine Biosynthetic (APB) Pathway in *Salmonella typhimurium*

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ABSTRACT

Thiamine is thought to be synthesized by two alternative pathways, one involving the first four enzymes of the purine pathway and a second that can function independently of the purine pathway. Insertion mutations in *purG* and *purI* prevent thiamine synthesis through the alternative pyrimidine biosynthetic (APB) pathway under aerobic but not anaerobic growth conditions. In contrast, point mutations in *purG* and *purI* caused one of three distinct phenotypes: Pur$^-$ Apb$^-$, Pur$^-$ Apb$^+$, or Pur$^+$ Apb$^-$. Analysis of these three mutant classes demonstrated two genetically separable functions for PurG and PurI in thiamine synthesis. In addition to their known enzymatic role in *de novo* purine synthesis, we propose that PurG and PurI play a novel, possibly nonenzymatic role in the APB pathway. Suppression analysis of Pur$^-$ Apb$^-$ mutants identified two new genetic loci involved in the APB pathway, *apbB* and *apbD*. We show here that mutations in *apbB* and *apbD* cause distinct, allele-specific suppression of the thiamine requirement of *purG* and *purI* mutants. Our results suggest that PurG and PurI and one or more components of the APB pathway may function as a complex needed for aerobic function of the APB pathway.

As the study of cellular metabolism progresses, it has become necessary not only to understand individual pathways but also to identify interactions between them and determine the role these interactions play *in vivo*. The biosynthesis of thiamine (vitamin B_1) in *Salmonella typhimurium* serves as an attractive model system for studying pathway interactions for two reasons. First, since thiamine is an essential vitamin, ability to grow in the absence of exogenous thiamine demonstrates endogenous synthesis. The low cellular requirement for this vitamin means that such growth can provide a sensitive *in vivo* assay for thiamine synthesis. Thus, unlike high flux biosynthetic pathways, very small changes in carbon flux can be seen as the difference between growth and no growth. Second, our work and that of others have identified a role for at least four metabolic pathways in thiamine biosynthesis, making it likely that metabolic cross-talk is important in this system.

Thiamine synthesis involves the phosphorylation and condensation of two independently synthesized moieties, THZ [4-methyl-5-(β-hydroxyethyl)thiazole] and HMP (4-amino-5-hydroxymethyl-2-methylpyrimidine) as shown in Figure 1. In *S. typhimurium* there are at least two pathways for HMP synthesis. The well-described pathway uses the first five enzymes of *de novo* purine synthesis (PurF, -D, -N, -G, and -I) to form 5′-phosphoribosyl-5-aminoimidazole (AIR) (Neuhard and Nygaard 1987). AIR is then converted to purines by the activity of additional *pur* gene products or to HMP via proposed *thi* gene products (Newell and Tucker 1968a,b; Estramareix and Therisod 1984; Neuhard and Nygaard 1987; Vander Horn *et al.* 1993).

Thiamine synthesis can occur independently of *pur* genes via the alternative pyrimidine biosynthetic (APB) pathway (Figure 1) (Downs 1992; Downs and Petersen 1994). Thiamine synthesis through the APB pathway is prevented by mutations in a number of loci, including: *apbA*, which is proposed to encode an enzyme in the APB pathway (Downs and Petersen 1994; M. E. Frodyma and D. M. Downs, unpublished results), *apbC* (*mrp*), encoding a protein of unknown function (L. Petersen and D. M. Downs, unpublished results), *gnd* or *zwf*, encoding enzymes of the pentose phosphate pathway (Nasoff *et al.* 1984; Rowley and Wolf 1991; Enos-Berlage and Downs 1996; Petersen *et al.* 1996), and *purR*, encoding a transcriptional repressor (Petersen *et al.* 1996; Rolfes and Zalkin 1988). Recently, we have found that mutations affecting the tricarboxylic acid cycle also influence thiamine synthesis through the APB pathway (J. L. Enos-Berlage and D. M. Downs, unpublished results).

The involvement of such a diverse group of pathways in thiamine synthesis is intriguing from a regulatory standpoint. Multiple inputs, especially those that use metabolites from other pathways, might be expected to increase the metabolic flexibility of a cell by ensuring that thiamine synthesis occurs under many different growth conditions. However, to benefit from this metabolic flexibility, the cell would have to regulate the flow

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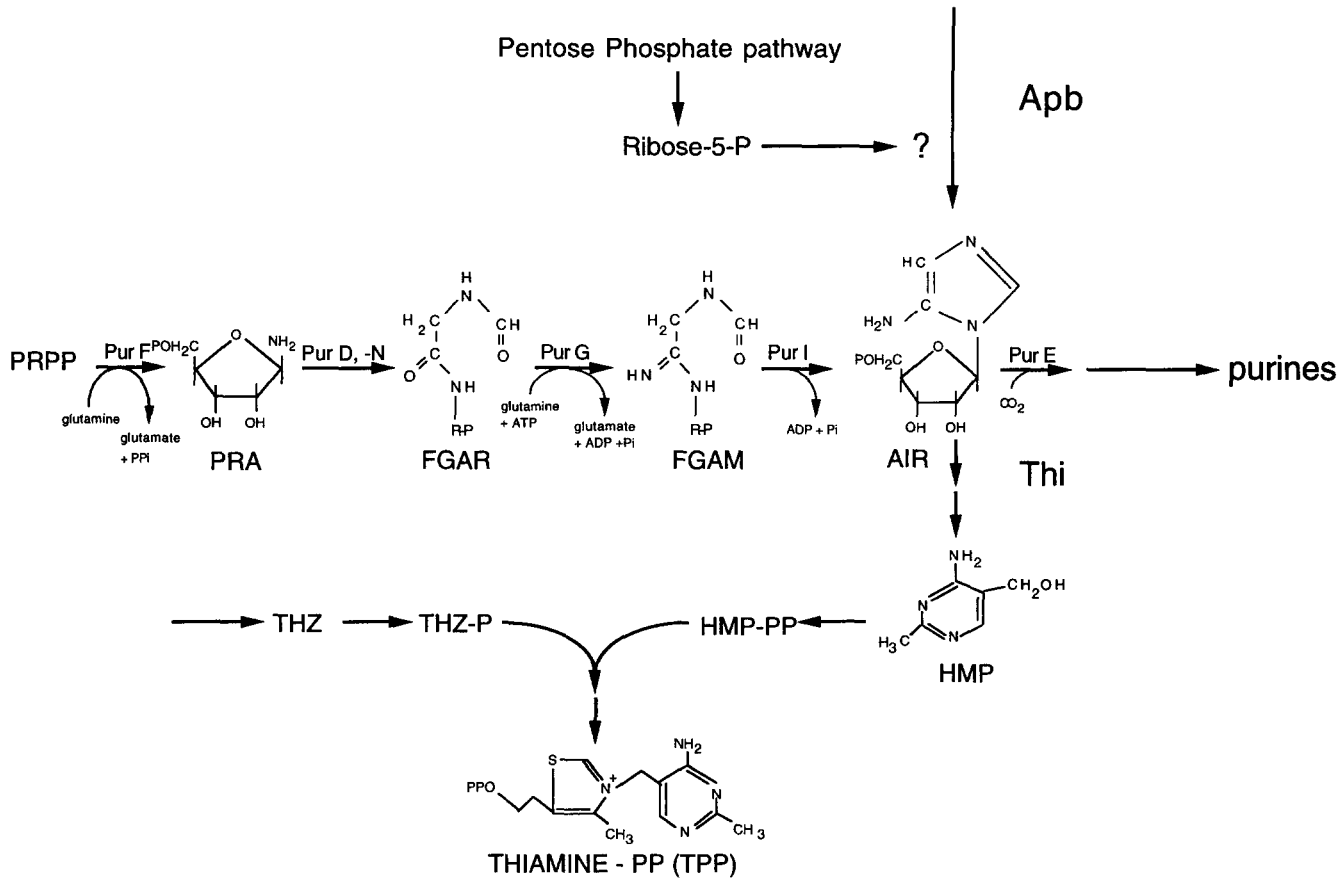

FIGURE 1.—Biosynthesis of thiamine. Synthesis of thiamine in *S. typhimurium* is depicted, highlighting the known roles of PurG and PurI in both purine and thiamine biosynthesis. The steps in the APB pathway and between AIR and HMP are currently unknown. APB, alternative pyrimidine biosynthetic; PRA, 5-phosphoribosylamine; FGAR, 5′-phosphoribosyl-*N*–formylglycinamide; FGAM, 5′-phosphoribosyl-*N*–formylglycinamidine; AIR, 5′-phosphoribosyl-5-aminoimidazole; THZ-P, 4-methyl-5-(β-hydroxyethyl)thiazole monophosphate; HMP-PP, 4-amino-5-hydroxyethyl-2-methylpyrimidine pyrophosphate.

of shared metabolites such that they were available to the appropriate pathway(s) under a given set of growth conditions. This regulation could be accomplished by branch point enzymes with different affinities for the same substrate, as has been shown for the branch point between the tricarboxylic acid cycle and the glyoxylate shunt (WALSH and KOSHLAND 1984). Alternatively, such regulation could involve metabolic channeling between sequential enzymes of a pathway or possibly between components of pathways that share metabolites. Pathways as diverse as protein synthesis, amino acid biosynthesis, and electron transport have been shown to involve multi-enzyme complexes that might facilitate such channeling (SRERE 1987). Genetic analysis provides a viable way to address pathway interactions and determine the mechanism(s) of metabolic cross-talk.

We report here an initial genetic analysis of the role PurG and PurI proteins have in thiamine synthesis in *S. typhimurium*. The work was initiated to address the observation that PurG and PurI proteins were required for thiamine independent growth under aerobic but not anaerobic conditions. We propose that PurG and PurI proteins have two distinct roles in thiamine synthesis: their previously described enzymatic role via *de novo* purine synthesis (NEUHARD and NYGAARD 1987) and a nonenzymatic, possibly structural, role in the APB pathway for thiamine synthesis.

MATERIALS AND METHODS

Bacterial strains and culture media: All strains used in this study are derivatives of *Salmonella typhimurium* LT2 and are listed with their genotypes in Table 1.

No-carbon E medium supplemented with 1 mM $MgSO_4$ (VOGEL and BONNER 1956; DAVIS *et al.* 1980) and a carbon source (11 mM) was used as a minimal medium, and Difco nutrient broth (8 g/liter) was used as a rich medium. Difco BiTek agar was added (15 g/liter) for solid medium. Adenine and thiamine were included in media as needed to the final concentrations of 0.4 mM and 0.5 μM, respectively. Antibiotics were added as needed to the following concentrations in micrograms per milliliter (rich/minimal): tetracycline (20/10) and kanamycin (50/125). All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Transductional methods: All transductional crosses were performed with the high-frequency, generalized transducing bacteriophage P22 mutant Ht*105/1, int-201* (SCHMIEGER 1972) as has been described previously (DOWNS and PETERSEN 1994). Transductants were purified by streaking on nonselec-

TABLE 1

Strain list

Strain	Genotype	Source[a]
LT2	Wild type	
TT317	*purF1741*::*Tn10d*(Tc)	J. ROTH
TT10288	*hisD9953*::MudJ[b] *his-9944*::Mud1	J. ROTH
DM40	*purG2324*::MudJ	Lab collection
DM42	*purI2152*::MudJ	Lab collection
DM44	*purD1874*::MudJ	Lab collection
DM493	*zfe-8016*::Tn*10d*(Tc)[c]	
DM494	*zfb-8017*::Tn*10d*(Tc)	
DM520	*zfe-8016*::Tn*10d*(Tc) *purG2936*	
DM521	*zfb-8017*::Tn*10d*(Tc) *purI2937*	
DM522	*zfb-8017*::Tn*10d*(Tc) *purI2938*	
DM526	*zfe-8016*::Tn*10d*(Tc) *purG2939*	
DM527	*zfe-8016*::Tn*10d*(Tc) *purG2940*	
DM528	*zfe-8016*::Tn*10d*(Tc) *purG2941*	
DM529	*zfb-8017*::Tn*10d*(Tc) *purI2942*	
DM530	*zfb-8017*::Tn*10d*(Tc) *purI2943*	
DM531	*zfb-8017*::Tn*10d*(Tc) *purI2944*	
DM532	*zfe-8016*::Tn*10d*(Tc) *purG2936 apbA1*::MudJ	
DM534	*zfb-8017*::Tn*10d*(Tc) *purI2938 apbA1*::MudJ	
DM539	*zfb-8017*::Tn*10d*(Tc) *purI2945*	
DM540	*zfb-8017*::Tn*10d*(Tc) *purI2946*	
DM541	*zfe-8016*::Tn*10d*(Tc) *purG2947*	
DM544	*zfe-8016*::Tn*10d*(Tc) *purG2941 apbA1*::MudJ	
DM545	*zfb-8017*::Tn*10d*(Tc) *purI2942 apbA1*::MudJ	
DM552	*zfb-8017*::Tn*10d*(Tc) *purI2948*	
DM553	*zfb-8017*::Tn*10d*(Tc) *purI2948 apbA1*::MudJ	
DM554	*zfe-8016*::Tn*10d*(Tc) *purG2949*	
DM1007	*zfb-8017*::Tn*10d*(Tc) *purI2944 apbB76*	
DM1216	*zfb-8017*::Tn*10d*(Tc) *purI2944 zje-8058*::MudJ *apbB76*	
DM1451-DM1468	*zfe-8016*::Tn*10d*(Tc) *purG2983-3000*	
DM1469-DM1484	*zfb-8017*::Tn*10d*(Tc) *purI3001-3016*	
DM1485-DM1502	*zfe-8016*::Tn*10d*(Tc) *purG2983-3000 apbA8* (Δ)	
DM1503-DM1518	*zfb-8017*::Tn*10d*(Tc) *purI3001-3016 apbA8* (Δ)	
DM1923	*zfb-8017*::Tn*10d*(Tc) *purI3006 apbD85*	
DM2093	*zfb-8017*::Tn*10d*(Tc) *purI3006 zxx-8087*::MudJ *apbD85*	
DM2103-DM2110	*zfb-8017*::Tn*10d*(Tc) *purI2944 zje-8058*::MudJ *apbB77-84*	
DM2148	*purI1757*::Tn*10*	
DM2149	*purG1739*::Tn*10*	
DM2182	*zfb-8090*::MudJ *purI3011*	
DM2213	*zfe-8016*::Tn*10d*(Tc) *purG3034*	
DM2215-DM2218	*zfb-8017*::Tn*10d*(Tc) *purI3036-3039*	
DM2220	*purF2085* (Δ) *zfe-8016*::Tn*10d*(Tc) *purG3034*	
DM2221-DM2224	*purF2085* (Δ) *zfb-8017*::Tn*10d*(Tc) *purI3036-3039*	
DM2230	*zfe-8016*::Tn*10d*(Tc) *purG2941 zfb-8090*::MudJ *purI3011*	
DM2238	*zfe-8016*::Tn*10d*(Tc) *purG2984 zfb-8090*::MudJ *purI3011*	
DM2277	*purF2085* (Δ) *zfe-8016*::Tn*10d*(Tc)	
DM2278	*purF2085* (Δ) *zfb-8017*::Tn*10d*(Tc)	

[a] Unless otherwise noted, all strains were constructed during the course of this work.
[b] MudJ refers to the defective transposon Mu dI*1734* (CASTILHO *et al.* 1984).
[c] Tn *10d*(Tc) refers to the transposition-defective mini-Tn*10* (Tn*10*del-16 del-17) (WAY *et al.* 1984).

tive green indicator plates and putative phage-free clones, identified by the light green color (CHAN *et al.* 1972), were verified to be phage-free by cross-streaking with phage P22.

Mutant isolation: *Pur⁻ mutants:* A P22 lysate was grown on strains carrying either *zfe-8016*::Tn*10d*(Tc) (46% linked to *purG*) or *zfb-8017*::Tn*10d*(Tc) (47% linked to *purI*). These phage lysates were subjected to hydroxylamine mutagenesis (HONG and AMES 1971; DAVIS *et al.* 1980) and used to transduce LT2 to tetracycline resistance (Tc^r). Tc^r transductants were replica printed sequentially to minimal glucose tetracycline plates and minimal glucose tetracycline plates with adenine and thiamine. Putative Pur$^-$ mutants were identified by their growth on only the latter plate. The *pur* mutations in these strains were transduced into a fresh genetic background by selecting Tc^r and scoring Pur$^-$.

Pur$^+$ Apb$^-$ mutants: In a separate experiment, Tc^r transduc-

tants generated as above were replica printed sequentially to minimal glucose tetracycline plates with no additions, adenine, and adenine and thiamine. Putative Pur$^+$ Apb$^-$ mutants were identified by growth on minimal media (Pur$^+$) and no growth on adenine unless thiamine was also supplied (Apb$^-$). Putative mutants were reconstructed by transduction into a wild-type genetic background. To confirm that these mutations caused an Apb$^-$ phenotype, they were transduced into a *purF2085* background. Transductants that inherited the *pur* point mutation from the donor were identified in each case by back crosses. In a *purF* background, an Apb$^-$ phenotype was defined as a thiamine auxotrophy on gluconate adenine medium (PETERSEN *et al.* 1996).

Phenotypic characterization: Phenotypes were assessed in liquid and on solid growth media.

Liquid growth curves: Appropriate strains were grown to full density in nutrient broth at 37°. After overnight incubation, cells were pelleted and resuspended in an equal volume of sterile saline (85 mM) and 0.1 ml of this suspension used to inoculate 5 ml of the appropriate medium. Culture tubes were placed in a shaking water bath at 37°, and growth was monitored as absorbance at 650 nm on a Bausch and Lomb Spectronic 20. Specific growth rate was determined as $\mu = \ln(X/X_0)/T$, where $X = A_{650}$ during the linear portion of the growth curve and T = time.

Plate tests: A 0.2-ml aliquot of a saline cell suspension prepared as above was added to 3.5 ml of molten 0.7% agar and spread on an appropriate plate. Nutrients to be tested were spotted after the agar had solidified. Ten microliters containing 80 nmol of adenine and 1 μl containing 0.1 nmol of thiamine were spotted as needed.

Reversion tests: Reversion frequencies were determined by plating 0.2 ml of a saline cell suspension (10^7 cells) on appropriate media and incubating at 37° for 2 days. Numbers reported are the average of at least two platings from independent cultures. Reversion of *purG* and *purI* insertion mutants was tested after overnight cultures were pelleted and concentrated 10-fold during resuspension in saline.

Strain constructions: To identify a MudJ linked to *apbB76,* a phage lysate grown on strain TT10288 (*hisD9953*::MudJ *his-9944*::Mud1) was used as previously described to generate a pool of cells containing MudJ insertions in the background of strain DM1007 (*purI2944 apbB76*) (HUGHES and ROTH 1988). Phage grown on the above pool was used to transduce DM531 (*purI2944*) to Km^r. Km^r transductants were screened for growth on minimal glucose adenine plates, implying inheritance of *apbB76.* Back crosses established that *zje-8058*::MudJ was 98% linked to *apbB76.*

Subsequently, *apbB* mutations were transduced into various genetic backgrounds by selecting Km^r and anticipating 98% of these transductants would also inherit the *apbB* allele from the donor. The ability of a *apbB* mutation to suppress the thiamine requirement of a purine mutant was scored by replica printing Km^r transductants to minimal media supplemented with adenine. If >70% of Km^r transductants were Thi$^+$, the respective *apbB* mutation was concluded to suppress the *pur* allele. A similar procedure was followed to identify a MudJ linked to *apbD85* (*zxx-8087*::MudJ, 50% linked to *apbD85*) and determine the *pur* alleles suppressed by mutations in *apbD.*

RESULTS

Initial observations: Strains carrying insertion mutations in the purine genes required for the formation of AIR (*purF, G, I*) grew anaerobically without the addition of thiamine due to function of the APB pathway (Table 2) (DOWNS 1992). The failure of these mutants to grow aerobically in the absence of thiamine was originally thought to reflect an oxygen sensitivity of the APB pathway (DOWNS 1992). Subsequent work showed that the APB pathway can function aerobically, but, under aerobic conditions, this pathway can only bypass the requirement for the PurF protein in thiamine synthesis (Table 2) (DOWNS and PETERSEN 1994; PETERSEN *et al.* 1996). We were intrigued by the observation that the PurD, -G, and -I proteins were required for thiamine synthesis aerobically but not anaerobically. Experiments presented here were initiated to address the involvement of PurG and PurI proteins in the APB pathway.

In our initial analysis strains carrying insertion mutations in *purD, -G,* or *-I* (DM44, DM40, and DM42, respectively) were phenotypically similar (Table 2), and lesions in *purN* were not available for testing. We have focused our current work on *purG* and *purI* because *purD* is in an operon with *purH,* which complicates nutritional analysis of these mutants. The results shown in Table 2 could be interpreted to mean that PurG and PurI were involved in the aerobic function of the APB pathway.

We hypothesized that PurG and PurI could either be required for APB pathway function or simply needed for maximal activity of the pathway. To address the latter possibility, we grew strains DM40 and DM42 (*purG2324*::MudJ and *purI2152*::MudJ, respectively) under conditions known to increase aerobic function of the APB pathway. These conditions included: introducing a *purE* mutation and growing cells on medium containing gluconate as a carbon/energy source (PETERSEN *et al.* 1996). Neither of these conditions allowed aerobic growth of DM40 or DM42 in the absence of thiamine, suggesting that PurG and PurI proteins were required for thiamine synthesis through the APB pathway under aerobic growth conditions.

Since none of the purine genes were required for anaerobic thiamine synthesis, it seemed unlikely that PurG or PurI proteins participated enzymatically in the APB pathway. If PurG or PurI proteins act by regulating the APB pathway, we expected mutations overcoming this regulation to arise as Pur$^-$ Thi$^+$ revertants of *purG* or *purI* insertion mutants. Although we have not rigorously ruled out this possibility, *purG* and *purI* insertion mutants (DM40, DM42) failed to revert to Thi$^+$ either spontaneously ($<10^{-10}$) or after diethyl sulfate mutagenesis.

We considered the possibility that the PurG and/or PurI proteins, but not their demonstrated enzymatic activities, were required for aerobic function of the APB pathway. This general model predicted the existence of point mutations in *purG* and *purI* genes that eliminate enzymatic activity yet still allow thiamine synthesis through the APB pathway. Strains containing the predicted mutations would be auxotrophic for purines while not requiring thiamine (Pur$^-$ Thi$^+$). Because the initial

TABLE 2

Phenotypes of *pur* insertion mutants

Strain	Genotype	Anaerobic growth			Aerobic growth		
		Min	ade	ade thi	min	ade	ade thi
TT317	*purF1741*::Tn*10d*(Tc)	−	+	+	−	+	+
DM40	*purG2324*::MudJ	−	+	+	−	−	+
DM42	*purI2152*::MudJ	−	+	+	−	−	+

[a] Growth was tested on solid NCE gluconate medium with noted additions (min, no additions; ade, adenine; thi, thiamine) after 24 hr at 37°. Anaerobic growth was performed in a gas chamber under an atmosphere of hydrogen and carbon dioxide.

steps in thiamine production can occur via the purine or the APB pathway, whenever thiamine synthesis in a strain is known to be dependent on the APB pathway we have described the strain as phenotypically Pur$^-$ Apb$^+$. Strains that are Pur$^-$ Apb$^-$ are implicitly Thi$^-$.

Identification of two separable functions for PurG and PurI: To identify the desired class of mutants (Pur$^-$ Apb$^+$), we used local mutagenesis to isolate strains containing point mutations in *purG* or *purI* genes. Phage lysates were grown on two strains containing either a Tn*10d*(Tc) 46% linked to *purG* (DM493) or one 47% linked to *purI* (DM494). In independent experiments, the lysates were mutagenized with hydroxylamine and used to transduce a wild-type strain (LT2) to Tc^r. Tc^r transductants were screened for those auxotrophs whose growth was restored by the addition of adenine and thiamine. With this screen, we identified 48 *pur* mutations (24 in *purG* and 24 in *purI*) without any bias for thiamine synthesis.

The 48 *pur* mutants fell into one of two classes based on their ability to grow in the absence of thiamine. Growth curves of a representative mutant from each phenotypic class are shown in Figure 2. Class I mutants were phenotypically Pur$^-$ Apb$^-$ (Figure 2A) and included strains carrying 12 *purG* and 17 *purI* point mutations (*purG2939, purG2940, purG2947, purG2949, purG2983, purG2985, purG2987, purG2989, purG2990, purG2995, purG2998, purG2999, purI2937, purI2943-6, purI3001, purI3002, purI3004-8, purI3010, purI3013-16*). These mutants were unable to grow in the absence of thiamine, displaying the phenotype previously reported for strains carrying insertion mutations in either of these two genes (Table 2).

Class II mutants were able to grow in the absence of thiamine (Pur$^-$ Thi$^+$), indicating that endogenous thiamine synthesis was occurring (Figure 2B). We considered two possibilities for the thiamine synthesis in class II mutants. A trivial explanation was that the *pur* mutations were lowering but not eliminating carbon flux through the purine pathway, resulting in a purine auxotrophy while allowing sufficient formation of AIR to supply the thiamine requirement. Alternatively, the mutations could have eliminated enzymatic activity of the respective Pur protein without eliminating its proposed function in the APB pathway. To distinguish between these possibilities, we blocked the APB pathway by transducing an *apbA* insertion mutation into each of the class II mutants.

In some cases (strains containing *purG2991-3, purI2942, purI2948, purI3003, purI3009*), the insertion mutation in *apbA* did not decrease the growth rate of the strain in the absence of thiamine (data not shown). This result was consistent with thiamine synthesis in these seven strains being derived from limited flux through the purine pathway and suggested the respective *pur* mutations resulted in partially functional enzymes. Consistent with this interpretation was the finding that six of these seven mutations permitted limited growth of the strain on minimal glucose medium (i. e., reached an A_{650} of 0.3 after 36 hr incubation at 37°, while insertion mutants reached an A_{650} of 0.1 after the same incuation). Since our data were consistent with these mutations resulting in leaky enzymes, these seven strains have not been pursued further and are not considered among class II mutants in the remainder of this manuscript.

Thiamine synthesis in the remaining 12 class II mutants (*purG2936, purG2941, purG2984, purG2986, purG2988, purG2994, purG2996, purG2997, purG3000, purI2938, purI3011*, and *purI3012*) was eliminated by an *apbA* insertion mutation. The inability of the *apbA* derivative strains to grow in the absence of exogenous thiamine is shown for a representative class II mutant in Figure 3. These results suggested that the class II mutants were using the APB pathway to produce thiamine despite the *pur* mutation.

The above analysis of class II mutants provided the first evidence that two functions of PurG and PurI in thiamine synthesis could be separated genetically. Based on this evidence, we pursued the hypothesis that PurG and PurI were involved in thiamine synthesis both through the previously described enzymatic role in *de novo* purine synthesis and through a new, possibly nonenzymatic role in the APB pathway.

Class I mutants can regain APB function: As stated above, class I point mutants displayed the same Pur$^-$ Apb$^-$ phenotype as was seen for insertion mutants. Further characterization showed that, unlike the insertion mutants, the point mutants gave rise to revertants able to grow on minimal medium at a frequency of $\sim 10^{-7}$

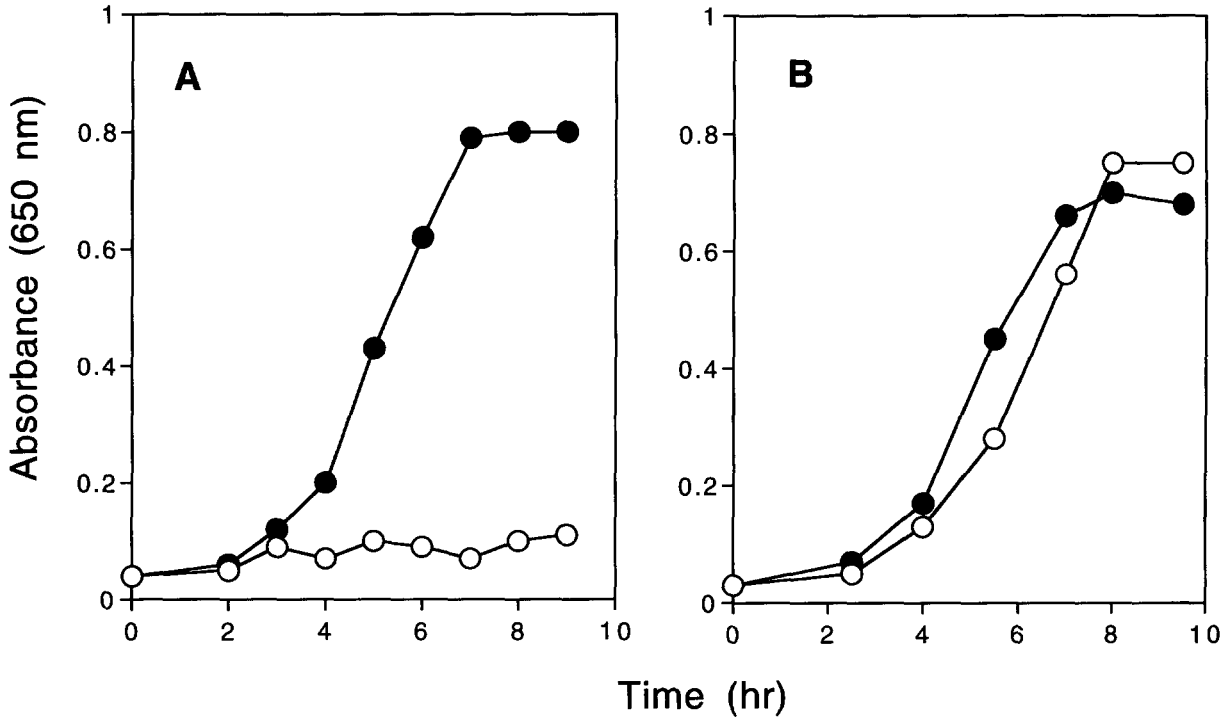

FIGURE 2.—Thiamine requirement of *pur* mutants. Representative aerobic growth curves of *pur* point mutants in glucose minimal media supplemented with adenine. (A) Class I, DM530 (*purI2943*). (B) Class II, DM520 (*purG2936*). ●, the same strain grown in the presence of exogenous adenine and thiamine.

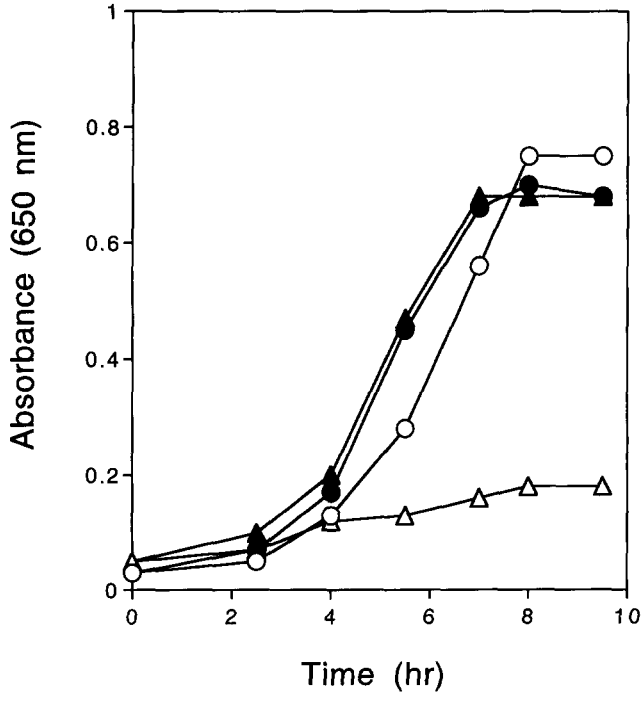

FIGURE 3.—Effect of an *apbA* mutation on the thiamine requirement of *pur* mutants. Representative aerobic growth curves of class II mutant DM520 (*purG2936*) (○) and its *apbA* derivative DM532 (△) in minimal glucose media supplemented with adenine (open symbols) or adenine and thiamine (solid symbols).

cell (Table 3). Transductional linkage analysis suggested that the 17 Pur$^+$ Thi$^+$ revertants we tested contained secondary mutations in the same gene as the original mutation (*i.e.*, *purG* or *purI*). We therefore assumed these revertants contained either a precise reversion event or an intragenic suppressor mutation.

Interestingly, some class I *purI* and *purG* point mutants (*e.g.*, DM531, *purI2944* in Table 3) reverted more frequently to growth on minimal adenine medium than on minimal medium. The higher frequency of reversion was due to appearance of a class of phenotypically Pur$^-$ Thi$^+$ revertants that could not have been detected in the absence of exogenous purines. We considered the possibility that thiamine synthesis in these revertants was due to restoration of APB pathway function. To test this possibility, we introduced an *apbA* insertion mutation into representative Pur$^-$ Thi$^+$ revertants of DM531 (*purI2944*). In all 15 cases tested, introduction of an *apbA* insertion mutation eliminated thiamine synthesis, strongly suggesting that the APB pathway was responsible for thiamine synthesis in the Pur$^-$ Thi$^+$ revertants. The phenotype of these revertants is therefore designated Pur$^-$ Apb$^+$ for the remainder of this report. After concluding that APB function had been restored in these Pur$^-$ Apb$^+$ revertants, we pursued the characterization of the causative mutations to gain insight on the role of the PurG and PurI proteins in APB-dependent thiamine synthesis.

TABLE 3

Reversion frequencies of class I mutants (Pur⁻ Thi⁻)

Strain	Relevant genotype	Revertants[a]	
		Minimal	Minimal ade
DM40	*purG2324::MudJ*	0	0
DM526	*purG2939*	0	0
DM527	*purG2940*	10	[b]
DM541	*purG2947*	10	[b]
DM554	*purG2949*	30	30
DM1451	*purG2983*	20	>80
DM1453	*purG2985*	3	7
DM1455	*purG2987*	3	3
DM1457	*purG2989*	10	10
DM1458	*purG2990*	20	20
DM1463	*purG2995*	2	3
DM1466	*purG2998*	6	>80
DM1467	*purG2999*	50	>80
DM42	*purI2142*::MudJ	0	0
DM521	*purI2937*	50	60
DM530	*purI2943*	10	3
DM531	*purI2944*	2	8
DM539	*purI2945*	4	9
DM540	*purI2946*	7	>80
DM1469	*purI3001*	0	0
DM1470	*purI3002*	0	0
DM1472	*purI3004*	3	20
DM1473	*purI3005*	[b]	4
DM1474	*purI3006*	2	7
DM1475	*purI3007*	2	>80
DM1476	*purI3008*	40	1
DM1478	*purI3010*	5	>80
DM1481	*purI3013*	[b]	2
DM1482	*purI3014*	7	4
DM1483	*purI3015*	2	4
DM1484	*purI3016*	[b]	1

Numbers shown are the average of at least two platings from independent cultures, similar trends were found in both platings. Colonies were counted after 48 hr incubation at 37°.

[a] Revertants shown per 10^7 cells. Approximately 10^7 cells in a saline suspension were spread on each plate.

[b] Reversion could not be quantitated due to high background growth.

Mutations in *apbB* display allele-specific suppression: To begin characterization of the Pur⁻ Apb⁺ revertants, it was necessary to transduce the responsible mutations into various genetic backgrounds. Since the secondary mutations in these revertants were allowing function of the APB pathway, we denoted the affected locus *apbB* and focused on a representative Pur⁻ Apb⁺ revertant, DM1007 (*purI2944 apbB76*). A MudJ pool was constructed in DM1007 and used to identify a MudJ (*zje-8058::*MudJ) 98% linked to *apbB76* (see MATERIALS AND METHODS). Transduction linkage analysis determined that *zje-8058::*MudJ was not linked to any *pur* gene or known *apb* gene. Eight additional independent Pur⁻ Apb⁺ revertants of DM531 (*purI2944*) also contained secondary mutations linked to *zje-8058::*MudJ. Together these nine mutations defined the *apbB* locus. Transductional linkage has placed this locus at approximately minute 54 on the chromosome, 6% linked to *cysK*.

We considered two general ways the *apbB* mutations could allow the APB pathway to function: by causing a general stimulation of the APB pathway, or by producing an altered product that restored an interaction with the mutant Pur protein. The first model predicted *apbB* mutations would have an effect on the thiamine requirement of many, if not all, *pur* mutant alleles, while the second scenario predicted the *apbB* mutations would suppress the thiamine requirement of specific *pur* alleles. To distinguish between these possibilities, the *apbB76* mutation was transduced into several class I mutants (Pur⁻ Apb⁻) and the ability of the resulting strains to synthesize thiamine was scored (see MATERIALS AND METHODS).

The *apbB76* mutation, which was isolated in a *purI* mutant background, did not restore thiamine independent growth in any of the 11 strains containing a point mutation in *purG* (Table 4). In contrast, *apbB76* suppressed the thiamine requirement in six of the 16 *purI* mutants. In each of the six strains, the *apbB76* derivative had a similar growth rate with and without thiamine, indicating full suppression of the thiamine requirement. The same pattern of strain-specific suppression was found when *apbB* alleles 77–84 (DM2103–2110) were tested. Although other explanations exist, this allele-specific suppression was consistent with a model invoking a complex between PurI and one or more components of the APB pathway.

Mutations in *apbD* are also allele specific: In many cases of physical interaction, mutations selected to suppress different alleles show distinct patterns of suppression. In our system, this predicted that *apb* alleles selected to suppress the thiamine requirement of different *pur* mutant alleles might suppress a set of *pur* alleles distinct from those suppressed by mutations *apbB76-apbB84*. To address this prediction, we examined Pur⁻ Apb⁺ revertants of a second strain, DM1474 (*purI3006*). While DM1474 had a reversion pattern similar to that of DM531 (*purI2944*) (Table 3), the thiamine requirement of this strain was not suppressed by the *apbB* mutations selected in DM531 (Table 4). As with the *apbB* mutations, thiamine synthesis in the Pur⁻ Apb⁺ revertants of DM1474 was dependent on function of the APB pathway (data not shown).

Transductional linkage determined that the secondary mutations in the Pur⁻ Apb⁺ revertants of DM1474 were not in *purI* or *apbB* and thus defined an additional locus, designated *apbD*. The procedure described above was used to identify a MudJ (*zxx-8087::*MudJ) 50% linked to the *apbD* mutation in DM1923 (*purI3006 apbD85*). Transduction of *apbD85* into several class I mutants revealed a pattern of suppression strikingly different from that generated by *apbB* alleles (Table 4). The Thi⁻ phenotype of 18 out of 27 *pur* point mutants

TABLE 4

Suppression of thiamine requirement by *apbB* and *apbD* alleles

Strain	Relevant genotype	Suppression	
		apbB76[a]	*apbD85*[b]
DM2149	*purG1739*::Tn*10*	−	−
DM526	*purG2939*	−	−
DM527	*purG2940*	−	+
DM554	*purG2949*	−	+
DM1453	*purG2985*	−	+
DM1455	*purG2987*	−	+
DM1456	*purG2988*	−	+
DM1457	*purG2989*	−	+
DM1458	*purG2990*	−	−
DM1460	*purG2992*	−	+
DM1463	*purG2995*	−	+
DM1466	*purG2998*	−	−
DM2148	*purI1757*::Tn*10*	−	−
DM521	*purI2937*	−	+
DM530	*purI2943*	−	−
DM531	*purI2944*	+	−
DM539	*purI2945*	+	−
DM540	*purI2946*	−	−
DM1469	*purI3001*	−	−
DM1470	*purI3002*	−	−
DM1472	*purI3004*	−	+
DM1473	*purI3005*	+	−
DM1474	*purI3006*	−	+
DM1475	*purI3007*	−	−
DM1476	*purI3008*	+	−
DM1481	*purI3013*	+	−
DM1482	*purI3014*	−	+
DM1483	*purI3015*	+	−
DM1484	*purI3016*	−	−

[a] Mutants were considered suppressed by *apbB76* if >70% of Km[r] transductants were Thi[+] as described in MATERIALS AND METHODS. The same pattern of suppression was seen with *apbB* alleles 77-84. The *apbB76* mutation was isolated based on suppression of *purI2944*.

[b] Mutants were considered suppressed by *apbD85* if >25% of Km[r] transductants were Thi[+] as described in MATERIALS AND METHODS. The *apbD85* mutation was isolated based on suppression of *purI3006*.

was suppressed by mutations in one of the two *apb* loci, while no *pur* insertion mutants (DM2148 and DM2149) were suppressed by mutations in either *apbB* or *apbD*.

Two significant points can be made from the results shown in Table 4. First, no *pur* alleles were suppressed by both an *apbB* and by an *apbD* mutation. This nonoverlapping allele-specific suppression suggested that the class of *pur* mutant alleles suppressed was not simply a reflection of the severity of the initial *pur* defect. Second, unlike *apbB*, mutations in *apbD* were able to suppress some *purG* alleles. The distinct patterns of suppression shown by *apbB* and *apbD* mutations were consistent with a model invoking an interaction between Pur enzymes and components of the APB pathway, possibly encoded by the *apbB* and *apbD* genes.

Mutations in *purG* and *purI* can cause an Apb[−] phenotype: A model involving physical interaction between purine and APB enzymes might predict the existence of *purG* or *purI* alleles that disrupted the APB pathway without affecting purine synthesis (Pur[+] Apb[−]). There were two technical difficulties in isolating potential Pur[+] Apb[−] mutants in *purG* or *purI*. First, a functional purine pathway can produce sufficient AIR for thiamine synthesis, thus preventing us from screening for thiamine auxotrophs in a wild-type background. Second, the definitive APB phenotype is a thiamine auxotrophy in a *purF* background (PETERSEN *et al.* 1996). The need for a *purF* mutation in the background prevented a simple screen for the desired Pur[+] phenotype. To circumvent these problems, we drew on past work with *apbA* mutants, which showed that blocking the APB pathway in a wild-type background caused an adenine sensitivity (*i.e.*, a thiamine auxotrophy in the presence of adenine) (DOWNS and PETERSEN 1994). To identify mutations in *purG* and *purI* causing a putative Pur[+] Apb[−] phenotype, we used the hydroxylamine-mutagenized phage lysates described above to transduce the wild-type strain (LT2) to Tc[r]. We screened Tc[r] transductants for those that grew on minimal media (Pur[+]) but not when adenine was present (Apb[−]). Putative mutants were reconstructed and growth curves on minimal media revealed no defect in purine synthesis (data not shown).

We analyzed one adenine-sensitive mutant (DM2213) containing a mutation linked to *zfe-8016*::Tn*10d*(Tc) (near *purG*) and four (DM2215-DM2218) with mutations linked to *zfb-8017*::Tn*10d*(Tc) (near *purI*). The adenine-sensitive *purG* mutant was sixfold less common than Pur[−] mutants from the same transduction, while the adenine sensitive *purI* mutants were found at a frequency similar to Pur[−] *purI* mutants. To confirm that the adenine-sensitive phenotype was caused by a defect in the APB pathway, we tested the phenotype of the respective mutations in a *purF* mutant. The putative Apb[−] mutations were transduced into a *purF* background and the ability of the resulting strains to grow on adenine gluconate medium was assessed. All five mutations appeared to reduce growth of the *purF* mutant in the absence of thiamine (Table 5). The *purG* allele (Apb[−]) had the most dramatic effect, exhibiting a phenotype of similar severity to the phenotype caused by mutations in *apbA* (PETERSEN *et al.* 1996). These results were consistent with specific *purG* and *purI* point mutations preventing function of the APB pathway without affecting *de novo* purine synthesis.

Pur[+] Apb[−] mutations in *purG* and *purI* are dominant: To confirm that the Pur[+] Apb[−] mutants were actually defective in the appropriate *pur* gene, we introduced a plasmid containing a wild-type copy of the respective gene (*purG* or *purI*). The *purG* plasmid, p40, contained a 4.7-kb insert, of which 3.7 kb was the *purG* coding sequence (SCHENDEL *et al.* 1989), while the *purI*

TABLE 5

Apb⁻ phenotype of *purG* and *purI* mutants

Strain	Genotype	Growth rate[a]	
		ade	ade thi
DM2277	*purF2085 zfe-8016*::Tn*10d*(Tc)	0.69	0.74
DM2220	*purF2085 zfe-8016*::Tn*10d*(Tc) *purG3034*	0.09	0.50
DM2278	*purF2085 zfb-8017*::Tn*10d*(Tc)	0.50	0.40
DM2221	*purF2085 zfb-8017*::Tn*10d*(Tc) *purI3036*	0.28	0.40
DM2222	*purF2085 zfb-8017*::Tn*10d*(Tc) *purI3037*	0.46	0.57
DM2223	*purF2085 zfb-8017*::Tn*10d*(Tc) *purI3038*	0.38	0.65
DM2224	*purF2085 zfb-8017*::Tn*10d*(Tc) *purI3039*	0.66	0.73

[a] Liquid growth was performed as described in MATERIALS AND METHODS. Growth rates were calculated as $\mu = \ln(X/X_0)/T$. Adenine (ade) and thiamine (thi) were added to final concentrations of 0.4 mM and 0.5 μM, respectively.

plasmid, p42, contained a 3-kb insert, of which 1 kb was the *purI* gene (SMITH and DAUM 1986). Both p40 and p42 were identified based on their ability to complement *pur* insertion mutations in the respective genes and were confirmed by sequence homology to the appropriate *pur* gene from *Escherichia coli*. Surprisingly, the plasmids were unable to complement the Apb⁻ phenotypes of the above mutants. While this was not the result we expected, it does not eliminate our model since mutations affecting some types of complexes might be dominant. For example, if a mutant protein locked other components in a more stable but inactive complex, it might be difficult to titrate the mutant protein out. Although additional work is needed to definitively show that the Apb⁻ mutants contain alleles of the *pur* genes, we feel that the alternative explanation, *i.e.*, *apb* genes closely linked to both *purG* and *purI*, is unlikely.

DISCUSSION

These studies were initiated to address the observation that the PurG and PurI proteins were required for thiamine synthesis through the APB pathway under aerobic but not anaerobic growth conditions (PETERSEN *et al.* 1996). The results presented herein demonstrate two genetically separable functions of the PurG and PurI proteins in thiamine synthesis. While other interpretations exist, our results are most consistent with these proteins providing essential physical contacts for one or more components of the APB pathway.

An important conclusion of this work is that the demonstrated enzymatic roles of PurG and PurI in *de novo* purine synthesis are not required for aerobic function of the APB pathway. This conclusion was based on the isolation of a phenotypic class of *purG* and *purI* point mutants that were Pur⁻ Apb⁺. While we have not shown biochemically that the Pur⁻ Apb⁺ class of mutants have completely lost the respective enzymatic activity, within the resolution of our experiments, the activity was absent. Further, introduction of an *apbA* mutation prevented thiamine independent growth in these strains and thus supported our conclusion that the APB pathway was responsible for thiamine synthesis in these Pur⁻ Apb⁺ strains. Based on the isolation and characterization of this class of point mutants, we concluded that the enzymatic activity of neither PurG nor PurI was required for APB-dependent thiamine synthesis.

Support for two distinct roles for PurG and PurI proteins in thiamine synthesis was provided by the generation of mutations (through local mutagenesis of the *purG* and *purI* genes) that caused a Pur⁺ Apb⁻ phenotype. The existence of both Pur⁻ Apb⁺ and Pur⁺ Apb⁻ mutants showed there were two genetically separable functions for the PurG and PurI proteins in thiamine synthesis. The analyses of the *purG* and *purI* point mutants described in this report have initiated the study of a second, possibly nonenzymatic, role for the PurG and PurI proteins in the APB pathway.

The results of analyses addressing the role of PurG and PurI proteins in thiamine synthesis have led us to propose that these proteins are involved in a physical complex with one or more components of the APB pathway. We further suggest that the integrity of this complex must be maintained for aerobic function of the APB pathway but is not necessary for *de novo* purine synthesis. The first evidence for physical contacts came from the allele-specific suppression of the thiamine requirement of some *purG* and *purI* mutants (Pur⁻ Apb⁻). We identified mutations in two loci, *apbB* and *apbD*, that can suppress the thiamine but not the purine auxotrophy of specific *pur* point mutants. Furthermore, we showed that this suppression required the APB pathway, consistent with compensatory mutations restoring a physical interaction essential for APB pathway function. Although alleles of both *apbB* and *apbD* showed a broad specificity with respect to the purine alleles they suppressed, there was no overlap between the group of purine mutations suppressed by *apbB vs. apbD* mutations (Table 4). While other interpretations exist, we feel this allele-specific suppression is most consistent with the formation of an essential protein-protein interaction. The relatively high number of purine mutations that were suppressed by each *apb* allele may indicate a complex with a large contact area, multiple components, or loose associations. Based on our model, we predict the *apbB* and *apbD* genes encode components of the proposed complex and thus define new loci involved in the APB pathway. Continued analyses of these loci will further our understanding of both the APB pathway and the role *de novo* purine enzymes have in thiamine synthesis through the APB pathway.

In summary, this work provides the first evidence of

a novel role for the PurG and PurI proteins in thiamine synthesis distinct from their demonstrated enzymatic activities. Continued genetic and biochemical analyses of this role will clarify the mechanism of interaction between two distinct pathways that share a metabolite. If the physical complex proposed here is verified by further work, it could represent a novel regulatory mechanism the cell uses to protect and/or direct shared metabolites.

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