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Synthesis of Thiamine in *Salmonella typhimurium* Independent of the *purF* Function

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In *Salmonella typhimurium*, the first five steps in purine biosynthesis also serve as the first steps in the biosynthesis of the pyrimidine moiety of thiamine (vitamin B_1). Strains with null mutations of the first gene of purine-thiamine synthesis (*purF*) can, under some circumstances, grow without thiamine. This suggests the existence of an alternative pathway to thiamine that can function without the *purF* protein. To demonstrate the nature and map position of the *purF* mutations corrected, a fine-structure genetic map of the *purF* gene was made. The map allows identification of deletion mutations that remove virtually all of the *purF* gene, as defined by mutations. We describe conditions and mutations (*panR*) which allow B_1 synthesis to occur in deletion mutants lacking *purF* function. The alternative route of B_1 synthesis appears to require enzymes which act subsequent to the *purF* enzyme in the purine pathway.

The first step in the purine pathway is catalyzed by the enzyme phosphoribosyl PP_i amidotransferase (EC 2.4.2.14), which is encoded by the *purF* gene of *Salmonella typhimurium* and *Escherichia coli*. In the reaction catalyzed by this enzyme, phosphoribosyl PP_i receives an amino group and loses PP_i from glutamine to yield phosphoribosylamine (PRA). This enzyme (from *E. coli*) is subject to feedback inhibition by IMP, AMP, and GMP; the inhibitions by AMP and GMP are synergistic (23, 26). It has been postulated that the *purF* enzyme is also inhibited by aminoimidazole carboximide ribotide (AICAR) (25). It has been shown genetically in *Saccharomyces cerevisiae* that AICAR inhibits an early step in the purine pathway (21). The *purF* gene and the enzyme encoded by it from *E. coli* have been extensively characterized. The enzyme has been purified to homogeneity, and its activities have been analyzed (23, 31). The nucleotide sequence of the *purF* gene has been determined and used to deduce the amino acid sequence of the enzyme (17, 32). Regulation of the *purF* gene has been studied by operon fusions which join the *purF* promoter to a complete *lacZ* gene (17, 29) and by in vitro assay of the mRNA encoding the *purF* enzyme (17). A repressor gene has been identified in both *E. coli* and salmonellae (9, 22, 30a). However, there has been no extensive genetic characterization of mutations in the *purF* region. To pursue unexpected phenotypes of some *purF* mutants, we have initiated a genetic analysis of the *purF* region.

In a number of organisms, the first step in purine biosynthesis uses ammonia and ribose-5-phosphate instead of phosphoribosyl PP_i and glutamine to synthesize PRA. This alternative reaction has been shown to occur in enteric bacteria; it is known to occur nonenzymatically (26) and enzymatically (15). The alternative reaction in *E. coli* is inhibited by AMP or GMP with the same general kinetics as the glutamine-dependent formation of PRA (15). Since the purified enzyme catalyzes both reactions, it seems likely that both of the in vitro activities could be provided by a single protein (23). There has been no demonstration of the physiological importance of this alternative reaction seen in vitro.

The physiological role and regulation of *purF* activity is complicated by its involvement in the biosynthesis of vitamin B_1 (thiamine). The early reactions of the purine pathway also serve as the first five steps in the synthesis of the pyrimidine moiety of thiamine. Newell and Tucker showed both genetically and biochemically that aminoimidazole ribotide (AIR) is the last common intermediate in the two pathways (24, 25). With the exceptions noted below, purine auxotrophs blocked before AIR require both purine and thiamine while those blocked later in the purine pathway require only purine (see Fig. 1).

Some *Salmonella* mutants blocked after AIR (*purH* and *purJ*) show a nutritional requirement for thiamine when adenine or hypoxanthine serves as the purine source (35). Similar mutations were found in *E. coli* (16). An explanation of this phenomenon was devised by Newell and Tucker in the course of their work on B_1-purine interactions (25). They demonstrated that mutants blocked in either the *purH* or the *purJ* gene accumulate AICAR; they proposed that this accumulation causes feedback inhibition of *purF*, limiting production of AIR and thereby generating a thiamine requirement. The *purH* and *purJ* mutants listed require thiamine only when a sufficiently high concentration of adenine or hypoxanthine is provided as the source of purine, presumably because of an additive feedback inhibition effect of purine and AICAR. This phenotype might best be described as adenine inhibition of B_1 synthesis, since *purH* and *purJ* mutants synthesize thiamine when grown on low levels of adenine (7a).

The B_1 requirement of *purH* and *purJ* mutants can be satisfied by exogenous histidine. This surprising fact is actually consistent with the proposal of Newell and Tucker. The purine and histidine pathways are closely related (see Fig. 1), including a cycle of reactions that includes part of both biosynthetic reaction sequences. The initial substrate of the *his* pathway is ATP, and the purine biosynthetic intermediate AICAR is produced as a by-product of histidine synthesis (3). The cyclical nature of these pathways was emphasized in early chemical literature prior to complete elucidation of these pathways (16). The ability of histidine to suppress the thiamine requirement of *purH* and *purJ* mutants

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is thought to be due to feedback inhibition of the first enzyme of the histidine pathway, i.e., the *hisG*-encoded enzyme (20). It was proposed that feedback inhibition of the histidine pathway eliminates one source of AICAR and reduces the internal AICAR level. This reduction was thought to relieve AICAR inhibition of the *purF*-encoded enzyme and restore B_1 synthesis (10).

In the course of work on purine-histidine interactions, we have made several observations that suggest that AIR synthesis regulation has additional complexities. We present here genetic evidence for an additional route of AIR synthesis distinct from the known pathway. Synthesis of AIR through the new pathway can be enhanced by either mutation or the presence of exogenous pantothenate in the culture medium.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. All of the strains used in this study were derived from *S. typhimurium* LT2 and are listed with their sources in Table 1. Plasmid pCM33 was provided by H. Zalkin and contains a *Hin*cII fragment completely internal to the *purF* coding sequence (from *E. coli*) cloned into pUC8 (17, 36).

The E medium of Vogel and Bonner (33), supplemented with 0.2% glucose, was used as minimal medium. Difco nutrient broth (8 g/liter), with NaCl (5 g/liter) added, was used as rich medium. Difco Bacto-Agar was added to a final concentration of 1.5% for solid medium. The following additives were included in medium, as needed (final concentrations are given): tetracycline (15 μg/ml in rich medium), ampicillin (30 μg/ml), kanamycin (50 μg/ml), thiamine (0.05 mM), inosine (0.3 mM), pyridoxine-HCl (500 nM), histidine (0.1 mM), pantothenate (0.1 mM), and adenine (5 mM). High-adenosine medium contained 40 mM adenosine. Bochner medium was used to select tetracycline-sensitive derivatives from strains carrying Tn*10* (Tc^r); this medium was made as described previously (2, 18).

Detailed phenotypes of *purF* mutants were determined as follows. Cultures were grown to full density in nutrient broth containing added inosine. Cells were pelleted by centrifugation, and the pellet was suspended in an equal volume of saline. A 0.1-ml sample of this culture was added to 4 ml of 0.7% agar and poured onto a minimal plate with the appropriate nutrients added. A small sterile filter disk was placed in the center of the plate, and 20 μl of a solution of adenine (116 mM, pH ~3.5) was added to the disk. Growth was observed after 48 h at 30°C. The ability of mutants to excrete metabolites was tested as follows. A nutrient broth culture of the strain to be fed was centrifuged to pellet the cells. The cells were suspended in an equal volume of saline. A 0.1-ml volume of this culture was added to 4 ml of 0.7% agar and poured onto the appropriate plate. Single colonies from the strain to be tested for excretion were stabbed into the above-described plate. Feeding was positive if the lawn of cells grew around the stabbed cells.

Liquid growth curves were determined as follows. Nutrient broth cultures were pelleted and suspended in saline. A 1:25 inoculation was made into the appropriate medium. Cells were incubated with shaking at 30°C. Cell turbidity was monitored with a Bausch & Lomb Spectronic 20 spectrophotometer at 650 nm.

Transductional methods. The high-frequency generalized transducing mutant of bacteriophage P22 (HT105/1, *int-201*) (28) was used for all transductional crosses. Recipient cells (10^8 CFU) and transducing phage (10^8 to 10^9 PFU) were spread directly on selective plates. All crosses selecting Pur^+ recombinants were done with 8 to 12 h of preincubation on nutrient broth plates containing inosine (to allow nonselective growth), followed by replica printing onto minimal medium. Transductants were purified by streaking on nonselective green indicator plates and putative phage-free clones identified by their light-colored colonies (5). Possible

TABLE 1. Strain list

Strain	Genotype	Source
TT12232	*zeh-1887*::Tn*10*	
TT12235	*zeh-1887*::Tn*10 pdxB543*	
TT12233	*zeh-1888*::Tn*10d*(Tc)[a]	
TT12236	*zeh-1890*::Tn*10d*(Tc)	
TT7693	*hisF9954*::MudA	Laboratory collection
TT7690	*hisF9951*::MudA	Laboratory collection
TT7726	*purF2054*::MudA	Laboratory collection
TT12234	*zeh-1889*::Tn*10d*(Tc)	
TT317	*purF1741*::Tn*10*	Laboratory collection
TT11	*purI1757*::Tn*10*	Laboratory collection
TT311	*purD1735*::Tn*10*	Laboratory collection
TT315	*purG1739*::Tn*10*	Laboratory collection
TT12603-12605	*zeh-1887*::Tn*10 purF2059-2061*	
TT12606-12607	*zeh-1887*::Tn*10 purF2064-2065*	
TT12608-12610	*zeh-1887*::Tn*10 purF2067-2069*	
TT12611	*purF-2070*	
TT12612-12616	*zeh-1887*::Tn*10 purF2071-2075*	
TT12617	*purF2076*	
TT12618-12621	*zeh-1887*::Tn*10 purF2077-2080*	
TT12622-12642	*purF-2081-2101*	
TT12643-12644	*purF2111-2112*	
TT12645-12646	*zeh-1887*::Tn*10 purF2113-2114*	
TT12647	*zeh-1887*::Tn*10 purF2116*	
TT12648-12651	*zeh-1887*::Tn*10 purF2120-2123*	
TT12652	*zeh-1887*::Tn*10 purF2134*	
TT12653-12654	*zeh-1887*::Tn*10 purF2136-2137*	
TT12655	*purF2115*	
TT12656-12658	*purF2117-2119*	
TT12659-12662	*purF2124-2127*	
TT12663-12667	*purF2129-2133*	
TT12668	*purF2135*	
TT12669-12670	*purF2138-2139*	
TT12304	*purF2054*::MudJ	
TR6430	*aceE*	Laboratory collection
TR6832	*nadC423*	Laboratory collection
TR1639	*pan-355 hisD9645*	Laboratory collection
TT13732	DUP[b] ([*hemL335*] MudJ [*nadC220*])	T. Elliot
TT13733	DUP ([*proC691*] MudJ [*hemL335*])	T. Elliot
TR6988-6994	*panR553-559*	
TT13722	*purF2099 panR560*	
TT13723	*purF2085 panR561*	
TT13724	*zae-3653*::Tn*10 panR554*	
TT13725	*zae-3653*::Tn*10*	

[a] Tn*10d*(Tc) refers to the transposition-defective mini-Tn*10* described by Way et al. (34).

[b] DUP, constructed duplication made by recombination between MudJ elements.

phage-free colonies were checked for phage sensitivity by cross-streaking with phage P22.

Isolation of *purF* point mutations. Transducing phage was grown on a strain (TT12232) carrying a Tn*10* insertion near the *purF* locus. This lysate was mutagenized as previously described (7, 11) and used to transduce LT2 to Tc^r. The Tc^r transductants were tested for purine auxotrophy by replica printing to minimal plates containing tetracycline and minimal plates containing adenine, B_1, and tetracycline. Purine auxotrophs were picked and purified as described in the previous section. This procedure yielded 20 independent auxotrophs satisfied by adenine plus B_1, one auxotroph satisfied by pyridoxine hydrochloride (*pdxB*), and one auxotroph whose nutritional requirement was defined only by the fact that it grew on E medium supplemented with Casamino Acids. The latter mutant did not grow on any of the pool media designed to diagnose standard single auxotrophic requirements (7) and could represent a mutation in a previously undescribed gene in the region.

Some additional *purF* point mutations were isolated by transducing the *pdxB* auxotroph (TT12235) to Pdx^+ by using a mutagenized P22 lysate grown on LT2; this recipient strain carries a Tn*10* insertion near *purF* and a *pdxB* mutation which maps near *purF* and confers pyridoxine auxotrophy. Among Pdx^+ transductants, we found 21 Pur^- mutants, roughly half of which had lost the recipient Tn*10* insertion.

Isolation of *purF* deletion mutants. Several insertions of a transposition-defective derivative of Tn*10* [Tn*10d*(Tc)] that are linked to the *purF* gene were isolated (14, 34). Two of these, *zeh-1888*::Tn*10d*(Tc) (TT12233) and *zeh-1890*::Tn*10d* (Tc) (TT12236), were chosen for use in isolating deletions because of their high linkage (ca. 70% cotransduction) to *purF* point mutations. To select Tc^s derivatives (including deletions), independent cultures of these strains were grown in nutrient broth and plated on Bochner plates (2) as modified by Maloy and Nunn (18). As an additional modification, the fusaric acid was dissolved in dimethylformamide prior to addition to the molten solid medium; selection was carried out on the resulting plates at 42°C. This procedure provides positive selection for tetracycline-sensitive strains. Surviving colonies were patched to a master plate of Bochner medium and printed to minimal medium and minimal medium containing adenine and B_1. Independently isolated purine auxotrophs were single colony isolated and saved for characterization. Strain TT12233, which contains a Tn*10d*(Tc) insertion 68% cotransducible with $purF^+$, was used to isolate 19 Tet^s Pur^- deletions. Strain TT12236, which contains an independently isolated Tn*10d*(Tc) insertion 72% linked to $purF^+$, was used to generate four additional Pur^- deletions.

Genetic mapping. Cells to be used as recipients in *purF* mapping crosses were grown in nutrient broth supplemented with inosine. Transductional crosses were carried out as described above. The crosses involved 2×10^9 PFU of the transducing lysate and 4×10^8 recipient cells. After 2 days of incubation on minimal medium, recombinants were scored. A negative result represents a decrease of at least 10^3-fold in the number of recombinants compared with an unrestricted cross with a wild-type donor. The resolution of these mapping crosses is limited by the poor transducibility of many *purF* mutants. A *purF* mutation is transductionally repaired approximately 100-fold more poorly than a *trp* mutation in a strain containing mutations in both loci. Hfr mapping of *panR* mutations was carried out as described by Chumley et al. (6).

Orientation of *purF2054*::Mud insertion. Transposition-defective Mud-*lac* phages have been previously described (1, 12). The direction of transcription of the *purF* gene was determined as previously described by Hughes and Roth (13). Strain TT7693 (*hisF9945*::MudA) carries a *lac* operon transcribed by the *his* promoter causing the strain to be Lac^+. Strain TT7690 (*hisF9951*::MudA) carries a fusion in the same gene but in the opposite orientation (Lac^-). By having two Mud insertions known to be opposite in orientation, it is possible to orient a third Mud insertion since duplication formation can occur by recombination between Mud elements only if they are in the same orientation (13). Duplication formation is determined by scoring Ap^r prototrophs that arise when strain LT2 is transduced to Ap^r by using as a donor a mixture of lysates grown on two auxotrophic Mud insertion mutants. Inheritance of a Mud prophage (Ap^r) requires that two transduced fragments enter the recipient and recombine. If the two fragments are derived from insertions in the same orientation at two different sites in the chromosome, duplications can be formed. These duplications can be identified since they show neither of the auxotrophic requirements characteristic of the parental insertion mutants.

To orient *purF* transcription, a Lac^+ MudA insertion in *purF* (TT7726) was used as the insertion of unknown orientation. A transducing lysate of each of the known *his*::MudA insertion mutants was mixed with a lysate of the strain carrying *purF2054*::MudA. These two lysate mixtures (TT7726 plus TT7690 and TT7726 plus TT7693) were used to transduce LT2 to Ap^r. Only the mixed lysate containing *purF2054*::MudA (TT7726) and *hisF9951*::MudA (TT7690) produced prototrophic Ap^r transductants. Thus, we conclude that these two insertions are in the same orientation; since the effective *his* insertion was not expressed by the *his* promoter and the *purF* insertion was expressed by its promoter, we conclude the *his* and *purF* operons are transcribed in opposite directions. This means that the *purF* gene is transcribed counterclockwise.

To orient the genetic map with respect to transcription, duplications constructed in the above-described crosses (*purF-his*) were transduced to Tc^r by using a phage lysate on a strain containing *zeh-1888*::Tn*10d* (TT12233). The Tc^r Ap^r transductants in each case were tested for segregation of Tc^s Ap^s colonies. None of the transductants segregated Tc^s clones, although Ap^s clones arose at a detectable frequency (5%). This suggests that Tn*10* is not located inside the duplication formed between *purF* and *hisF*. Since *zeh-1888*::Tn*10d* was used to generate most of the deletions, it must be on the side of the map from which all of the deletions enter. Thus, the deletions extend clockwise from the *purF* gene and remove the promoter end of the gene.

Isolation of *panR* mutants. Strains containing *panR* mutations were isolated in two ways. Nutrient broth cultures of strain TT12640 (deletion *purF2099*) were pelleted, and the cells were suspended in an equal volume saline. Equal (0.1-ml) volumes of this suspension were plated on minimal plates containing 5 mM adenine. Clones containing *panR* mutations appeared as B_1-independent colonies feeding the background *purF* lawn. Cultures of a *purH355* mutant strain were treated in a similar manner but plated on minimal plates containing 40 mM adenosine. Under these plating conditions, *purH* mutants require B_1 (see Introduction). Again, *panR* mutants appear as B_1^+ clones feeding the B_1 requirement of the background lawn.

Southern hybridizations. Chromosomal DNA was isolated as previously described (8). Plasmid purification and South-

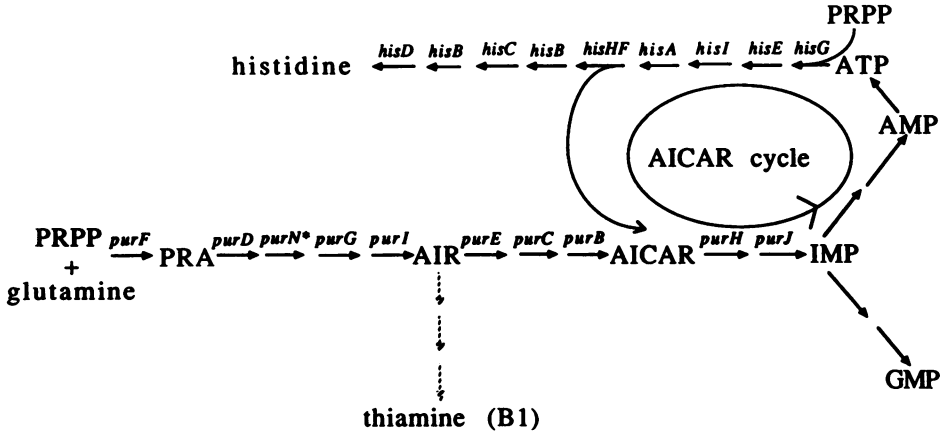

FIG. 1. Purine and histidine biosynthetic pathways. Both the purine and histidine biosynthetic pathways and their connection through the AICAR cycle are represented. Each arrow represents a single enzymatic step which is catalyzed by the product of the gene indicated above the arrow. The gene designated *purN* (*) has been described only in *E. coli* (30); the analogous gene has not been characterized in salmonellae. The number of enzymatic steps required for biosynthesis of thiamine has not been determined. PRPP, phosphoribosyl PP_i.

ern blot hybridizations were performed as described by Maniatis et al. (19).

RESULTS

Initial observations. According to the pathway in Fig. 1, all *purF* mutants are expected to require B_1. We observed that some of the *purF* mutants in our laboratory collection do not require vitamin B_1. This was initially explained by suggesting that these alleles are slightly leaky, producing enough PRA to provide for B_1 synthesis but not enough for purine synthesis. When we observed *purF* insertion mutants with this phenotype, we suspected the existence of an alternative to *purF* function since insertions usually result in complete loss of functional protein. We proceeded to isolate more *purF* mutants and construct a genetic map that would allow identification of deletion mutants and test the possibility that a mutation's B_1 phenotype might correlate with the position of the mutation in the gene.

Construction of a genetic map of *purF*. The genetic map in Fig. 2 represents the results of transductional crosses between *purF* deletion mutants (used as recipients) and point mutants (used as donors). Point mutants were isolated by local mutagenesis using hydroxylamine as described in Materials and Methods. Deletion mutants were isolated by selecting Tc^s derivatives of a strain carrying a Tn*10d*(Tc) element inserted near the *purF* gene (2, 18). The low efficiency of transduction (see Materials and Methods) significantly reduced the achievable resolution of the map. The maximum resolution of the map presented is 10^3; that is, failure of two mutations to recombine represents a $>10^3$-fold reduction in the recombinant frequency compared with that seen with a wild-type (*purF*$^+$) donor. Southern hybridization analysis confirmed that *purF2099* removed at least the inter-

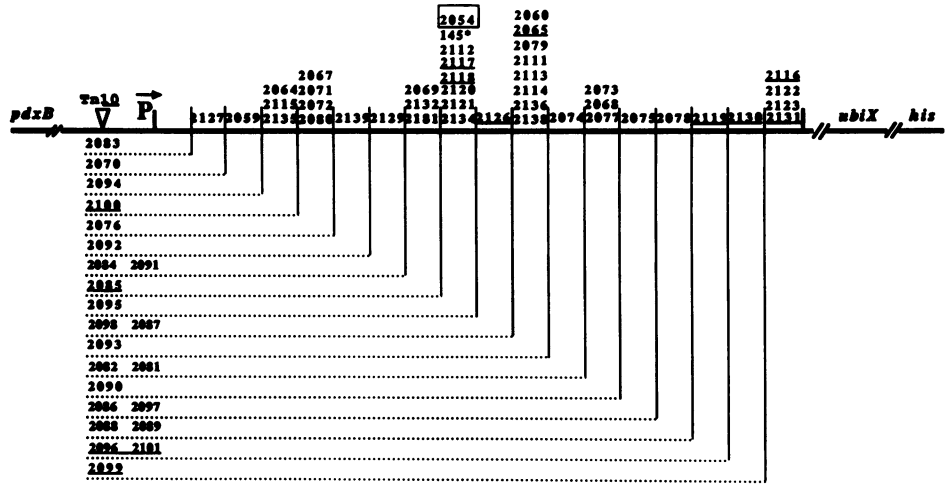

FIG. 2. Deletion map of *purF*. Deletions were generated from Tn*10* insertions as described in the text. Each mutation (deletion and point) is independent. The direction of transcription and neighboring genes are indicated above the map. The orientation of both is described in the text. Mutation *purF145* (*) is thought to be a small deletion, since it does not revert to Pur^+; however, this was not demonstrable by the crosses done here. Mutation *purF2054* (boxed on the map) is a Mud::*lac* fusion. The insertion element near the promoter is *zeh-1888*::Tn*10d*(Tc). Underlined alleles represent mutants with a B_1^- phenotype.

nal sequences of the *purF* locus. That is, DNA from strain TT12640 showed no sequences homologous to plasmid pCM33, which carries an internal fragment of the *E. coli* version of the *purF* gene (17, 36). The wild-type $purF^+$ control strain possessed the expected homology (data not shown). From these results we conclude that the gene and mutations studied in *S. typhimurium* do in fact correspond to the *purF* gene of *E. coli*.

Orientation of the *purF* map and transcription. The *purF* gene is transcribed counterclockwise on the *Salmonella* chromosome map and from left to right on the deletion map, as presented in Fig. 2. This was shown by first orienting transcription. An expressed (Lac^+) Mud-*lac* fusion (*purF2054*::MudA) was shown to be located in the same orientation as an unexpressed (Lac^-) Mud-*lac* fusion in the *his* operon (see Materials and Methods). Thus, transcriptions of *purF* and *his* genes must proceed in opposite directions. Since *his* transcription is clockwise, *purF* transcription must be counterclockwise.

Orientation of the deletion map (Fig. 2) in the chromosome was determined by finding that duplications formed by recombination between a *purF*::Mud insertion and a *his*::Mud insertion do not include the Tn*10* insertion at the left side of the *purF* region (*zeh-1888*::Tn*10d*). Thus, this Tn*10* element and the left end of the map (in Fig. 2) must be farthest from the *his* operon.

Variable B_1 requirement of *purF* mutants. While all of the *purF* mutants described here require a purine source, they exhibit a wide range of phenotypes with respect to the requirement for vitamin B_1. Phenotypic tests were done by supplying a source of adenine in the center of the plate (see Materials and Methods); this test was done with and without B_1 in the medium. In this test, each plate has a gradient of adenine concentration due to radial diffusion of adenine from the central disk. Most of the mutants (58 of the 71 tested) showed some B_1-independent growth. In some cases, the growth was seen only at low adenine concentrations. Less than half of the mutants (13 of the 71 tested) showed the phenotype expected for *purF* mutants, that is, a complete requirement for vitamin B_1. The difference between some *purF* mutants can be demonstrated in liquid culture. In Fig. 3, the open symbols represent growth of a B_1^+ (*purF2090*) mutant and the closed symbols show the growth phenotype of a B_1^- (*purF2085*) mutant (no detectable growth without added B_1).

The alternative pathway circumvents only the need for *purF* activity. Five known purine enzymes are involved in the reactions leading to synthesis of AIR, the branch point to B_1 synthesis (Fig. 1). Various mutants were tested for the ability to revert to B_1^+ (Table 2). Strain TT317 (*purF1741*::Tn*10*) reverts to B_1^+ at a frequency of 5×10^{-5}, compared with a revertant frequency of less than 10^{-9} for insertions in the other early purine genes. (The single revertant clone seen for one non-*purF* mutant had become Ade^+ B_1^+ Tc^s, presumably by precise excision of the Tn*10* element.) Since all *purF* mutants and only *purF* mutants show these reversions, the results suggest that the other purine genes are required for the alternative route of vitamin B_1 synthesis. To test this more directly, a *purG*, *purD*, or *purI*::Tn*10* mutation was introduced into two B_1^+ *purF* deletion mutants. Each of the introduced insertion mutations prevented B_1 synthesis (and reversion to B_1^+) (Table 3). This further shows that under the conditions tested, purine synthetic steps between PRA and AIR are required for the alternative route of B_1 synthesis; only the *purF* step can be circumvented.

B_1^+ phenotypes of *purF* mutations do not correlate with the *purF* genotype. There was no correlation between the position of the *purF* mutation in the genetic map and the ability of the mutant strain to make thiamine (Fig. 2). Since some deletion mutants grow without B_1, the ability does not appear to be due to leaky mutations leaving residual *purF* function. The mutations that allow B_1 production do not damage any particular part of the *purF* gene.

This variety of phenotypes and the absence of correlation with mutation type suggested that each strain might have acquired an independent secondary mutation which deter-

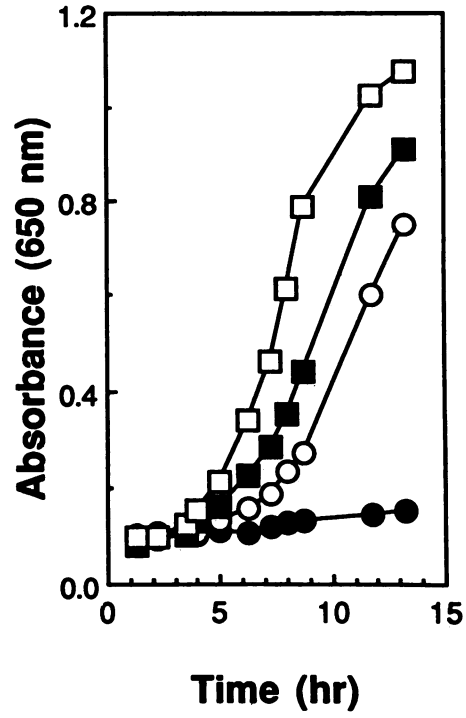

FIG. 3. B_1-independent growth. The growth of *purF2090* (B_1^+) (□ and ○) and *purF2085* (B_1^-) (■ and ●) mutants is shown. The medium in all cases was basal E medium containing adenine either with (□ and ■) or without (○ and ●) B_1. Growth occurred with shaking at 30°C.

TABLE 2. Frequency of B_1^+ revertants

Strain	Relevant genotype	No. of B_1^+ revertants/10^9 cells[a]
TT317	*purF1741*::Tn*10*	2×10^4
TT11	*purI1757*::Tn*10*	1[b]
TT311	*purD1735*::Tn*10*	0
TT315	*purG1739*::Tn*10*	0
TT12626	*purF2085*	10[c]
TT12642	*purF2101*	1×10^3
TT12641	*purF2100*	750
TT12640	*purF2099*	10[b]
TT12637	*purF2096*	1×10^3

[a] Reversion tests were done as described in Materials and Methods. Cultures were started from a single colony and grown in nutrient broth containing inosine.

[b] This colony was Ade^+ and no longer Tc^r, most likely because of precise excision of the Tn*10* element.

[c] This strain also had 50 to 100 small colonies on the plate. These colonies never grew to full size and were not stably propagated.

TABLE 3. Dependence of thiamine synthesis on the purine biosynthetic pathway[a]

Strain	Genotype	Growth on plates containing adenine and:	
		No further addition	B_1
TT11	*purI1757*::Tn*10*	−	+
TT315	*purG1739*::Tn*10*	−	+
TT12632	*purF2091*	+	+
TT12623	*purF2082*	+	+
TT12677	*purF2091 purI1757*::Tn*10*	−	+
TT12678	*purF2091 purG1739*::Tn*10*	−	+
TT12679	*purF2082 purI1757*::Tn*10*	−	+
TT12680	*purF2082 purG1739*::Tn*10*	−	+

[a] We plated 0.1-ml volumes of full-density cultures in top agar as described in Materials and Methods. A 20-μl volume of a 2% (wt/vol) solution of adenine was supplied in the center of the plate on a sterile filter disk. A plus sign indicates a zone of growth around the adenine source. Phenotypes were tested at 30°C.

mines its final B_1 phenotype. To test this, several *purF* deletions were transduced into a common genetic background by selecting for repair of a *pdx* mutation linked to the *purF* gene. P22 lysates on *purF2085* (II), *purF2076* (I), *purF2086* (I), and *purF2082* (I) were used to transduce TT12235 (Pdx^-) to Pdx^+ on E plates containing adenine and B_1. In such a cross, approximately 50% of the Pdx^+ transductants are Pur^-. From each cross, 10 Pur^- recombinants were purified and checked for their B_1 requirements (Table 4). Regardless of the phenotype of the donor deletion, Pur^- recombinants from each donor showed a distribution of phenotypes with respect to their B_1 requirements. The B_1^- transductants also varied in the frequency (over 100-fold) with which they reverted to B_1^+ (data not shown). This demonstrates that the variation in phenotype is not due to differences at the *purF* locus but is consistent with the idea that each transductant clone acquires a mutation affecting its vitamin B_1 phenotype after the deletion is introduced.

Attempts to map the inferred secondary mutations that confer thiamine synthesis were unsuccessful because of the variable phenotypes of the recombinants that emerged from crosses. Attempts to isolate mutations that stably inactivate the ability of a B_1^+ strain to synthesize thiamine were foiled by cross-feeding of thiamine by parental cells. Therefore, we concentrated our efforts on derivatives of B_1^- strains that had acquired the ability to make B_1 by a deliberately selected reversion event. Among the B_1^+ revertants were many colonies that appeared to be unstable as judged by our inability to propagate them stably on selective (E plus adenine) plates; however, some B_1^+ revertants formed large colonies and appeared to excrete something into the medium that fed the parental lawn of B_1^- cells. These revertants were stable and owed their B_1^+ phenotype to mutations that could be manipulated easily. This will be described later.

TABLE 4. Phenotypic dependence on background

purF allele in donor	Phenotypic class of donor	No. of Pur^- recombinants with the following phenotype[a]:	
		B_1^+	B_1^-
2076	I (B_1^+)	3	7
2086	I (B_1^+)	5	5
2082	I (B_1^+)	3	7
2085	II (B_1^-)	1	9

[a] Strain TT12235 (*pdx*) was transduced to pdx^+ by using phage grown on the appropriate donor strain. Transductants (pdx^+) were screened for their phenotypes. Ten Pur^- colonies from each cross were single colony isolated and classified by B_1 requirements as described in Materials and Methods.

Mutations that overexpress the alternative route of thiamine synthesis. Two selections were used to isolate mutants that overexpress the alternative pathway. First, B_1^+ revertants of B_1^- *purF* deletions were selected. At a frequency of ca. 10^{-7}, large B_1^+ revertant colonies surrounded by a halo of growth of the background (B_1^-) lawn appeared. These revertants excreted a compound able to satisfy the B_1 requirement of the lawn of *purF* parental cells. The feeding phenotype of these mutants was stable and easily scored, allowing genetic manipulation of the suppressor mutation involved.

A second selection for isolation of mutants that overexpress the alternative pathway was based on the known regulatory interaction between the purine and histidine biosynthetic pathways (see Introduction). A *purH* mutant will not grow on high concentrations of adenine or adenosine (presumably because of feedback inhibition of *purF*, causing starvation for B_1). Among the mutations allowing B_1-independent growth of a *purH* mutant with high levels of purine are feeders similar to the revertants of *purF* mutants described above. The feeding *purH* revertants also arose at about 10^{-7}.

The mutations isolated in each of these selections behaved similarly in the characterizations described below. We have called the locus defined by these mutations *panR* for reasons that will be outlined below. Although *purF panR* double mutants make their own B_1, they still require purine and do not revert spontaneously to purine-independent growth (fewer than 10^{-10} cells). This indicates that *panR* mutations satisfy only the B_1, not the purine, requirement of *purF* mutants.

To characterize the phenotypes of *panR* mutants, the original *pur* mutations (*purH* or *purF*) in each of the strains were removed by transducing the strains to Pur^+ by using a phage lysate grown on wild-type cells. The resulting *panR* pur^+ strains still excrete a compound that feeds the B_1 requirement of a *purF* or *purH* mutant (under conditions of adenine-induced B_1 starvation). However, a *panR* mutant does not feed the B_1 requirement of a strain blocked in *purD*, *purG*, or *purI* or feed a thiamine auxotroph (*thi*) blocked later in the thiamine pathway. These results strongly suggest that the excreted compound is not B_1 itself but rather PRA or some compound that stimulates PRA formation. This suggestion is supported by the fact that a *purD panR* double mutant has the same feeding properties as the *panR* parent, demonstrating that synthesis of the excreted compound does not require purine biosynthetic enzymes.

The possibility that PRA itself is excreted seems unlikely in view of the findings of Schendel et al. (27). They have reported that PRA is chemically unstable and decomposes in aqueous solution, with a half-life of 38 s at 37°C. Chemical instability and the attached phosphate make it difficult to imagine that PRA could be excreted and then taken up and used efficiently by other cells. It seems unlikely that PRA, per se, is the excreted compound.

Mapping of *panR* mutations. A Tn*10* insertion linked to *panR* was isolated as previously described (14). A strain (TT13725) carrying such a Tn*10* insertion (30% linked by transduction to $panR^+$) was used for Hfr mapping by the method of Chumley et al. (6). This Tn*10* was located

between 3 and 7 min on the *S. typhimurium* linkage map (data not shown). Auxotrophic markers in this region (*nadC*, *aceEF*, and *pan*) were transduced to prototrophy by using a phage lysate grown on TT13725. Prototrophic transductants were scored for Tc^r to score linkage. The Tn*10* insertion (*zae-3653*::Tn*10*) proved to be 30% cotransducible with the *pan-355* mutation and unlinked (less than 1% cotransducible) to *nadC* and *aceE*.

Pantothenate is excreted by *panR* mutants. To demonstrate that the *panR* mutation (as well as the nearby Tn*10*) was linked to the *pan* locus, a phage lysate on TR6991 (*panR556*) was used to transduce pantothenate auxotrophic mutant *pan-355* to prototrophy. More than 95% of the Pan^+ transductant colonies fed the background lawn of Pan^- cells. This suggested to us that the *panR* mutation is extremely close to the auxotrophic *pan* mutation and causes excretion of a compound that satisfies the nutritional requirement of a pantothenate auxotroph.

Two subsequent results supported this idea. By using the feeding assay described in Materials and Methods, we were able to show that *panR* mutants feed the pantothenate requirement of TR1639 (*pan-355*). We also demonstrated that exogenous pantothenate can feed B_1^- *purF* mutants and also satisfy the B_1 requirement of *purH* mutants under B_1 starvation conditions (high purine concentration). Pantothenate cannot substitute for the B_1 requirement of a *purD*, *purG*, *purI*, or *thi* mutation. In addition, B_1 cannot feed the nutritional requirement of a pantothenate auxotroph (data not shown). It is interesting that prior to the genetic classification of purine mutants, Yura observed that pantothenate could substitute for B_1 in some "adthi" mutants (35). Presumably, these mutants were what we now know as *purF*, *purH*, or *purJ* mutants.

DISCUSSION

Work presented here was initiated to pursue the observation that *purF* mutations have variable phenotypes with respect to the requirement for vitamin B_1. A fine-structure deletion map of the *purF* gene was constructed. In building this map, we observed the following. (i) Mutations in *purF* (including deletions) fall into two broad phenotypic classes, i.e., those capable and those not capable of B_1 synthesis. There appear to be subtle factors which vary the extent of B_1-independent growth in the former class. Mutants in the common steps of the purine-thiamine pathway (including *purF*) are expected to require both B_1 and purine. Thus, *purF* deletion mutants that are capable of B_1 synthesis are surprising. (ii) The variation in the phenotype of *purF* deletion mutants does not correlate with the map positions of the lesions. The data presented here make it unlikely that only leaky *purF* mutants are B_1^+, since many large deletions synthesize B_1 and all deletions remove the promoter end of the gene and presumably block transcription of the remainder of the gene. (iii) The phenotype of *purF* deletions is not an inherent consequence of the *purF* mutation but depends on the genetic background. When a *purF* deletion is moved into a new strain, its original phenotype is not necessarily retained but a variety of phenotypes are seen among the transductants obtained from a single donor deletion.

Our data strongly suggest that there is an alternative way of forming PRA (product of the *purF* enzyme). The fact that every *purF* deletion we obtained (Ade^- and B_1^-) can revert to stable B_1-independent growth is strong genetic support for this idea. Since these revertants appear to require later *pur* genes (*purD*, *purG*, and *purI*) to make thiamine, we presume that PRA, generated by the alternative route, is converted by these enzymes to AIR. We have isolated revertants of *purF* mutants which overproduce pantothenate. It is difficult to envision the biochemical conversion of pantothenate to PRA (4). We think it is more probable that pantothenate acts a cofactor or regulator of the proposed alternative pathway to PRA.

It is interesting that while both the *panR* mutation and exogenous pantothenate can satisfy the B_1 requirement of *purF* mutants, they do not satisfy the purine requirement. The fact that pantothenate does not satisfy the thiamine requirement of *purD*, *purG*, or *purI* gene mutants suggests that it stimulates production of PRA, which can be converted to AIR. As shown by Schendel et al. (27), PRA is chemically unstable. Therefore, only a small proportion of the PRA formed in the presence of pantothenate may actually enter the purine pathway. It may not be possible for the alternative pathway to form enough PRA to satisfy the purine requirement, which is significantly larger than the B_1 requirement.

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