Elevated Levels of Ketopantoate Hydroxymethyltransferase (PanB) Lead to a Physiologically Significant Coenzyme A Elevation in *Salmonella enterica* Serovar Typhimurium

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Pantothenate is the product of the ATP-dependent condensation of pantoate and β -alanine and is a direct precursor of coenzyme A. A connection exists between pantothenate biosynthesis and thiamine biosynthesis in *Salmonella enterica* serovar Typhimurium since derivatives of a *purF* mutant that can grow (on glucose medium) in the absence of thiamine excrete pantothenate. We show here that the causative mutation in three such mutants was the addition of a CG base pair upstream of the *panB* gene. This base addition brings the spacing between the -10 and -35 hexamers of the promoter to a consensus spacing of 17 bp and results in increased transcription of the *pan* operon. Furthermore, overexpression of PanB caused by this mutation, or by other means, was necessary and sufficient to increase pantothenate production and allow PurF-independent thiamine synthesis on glucose medium.

Pantothenate is an essential cofactor that is synthesized de novo in *Salmonella enterica* serovar Typhimurium by a well-characterized pathway (Fig. 1A) (7, 16). Three of the four biosynthetic genes (*panBCD*) are clustered at 3 min (7, 20), while the fourth is in a single gene locus at 10 min on the *Salmonella* and *Escherichia coli* chromosome maps (12). Transcriptional regulation of the pantothenate biosynthetic genes has not been reported.

The only known role of pantothenate in metabolism is its conversion to phosphopantotheine, the acyl group carrier of coenzyme A (CoA) and acyl carrier protein (16). The enzymes required for conversion of pantothenate to CoA in *E. coli* have been identified (Fig. 1B) (1, 13, 16, 24, 32). Genetic characterization of the CoA biosynthetic pathway has been slowed by the fact that CoA is essential and intermediates in the pathway are not taken up by bacterial cells. Unlike pantothenate synthesis, CoA levels in the cell are tightly regulated. This regulation is achieved primarily by the allosteric inhibition of pantothenate kinase (CoaA) by CoA and its thioester derivatives (16, 18, 19, 28–30, 34, 35). One consequence of this regulation is that increasing endogenous pantothenate results in a less-than-twofold increase in CoA levels and efflux of the excess pantothenate from the cell (1, 7, 19, 22, 28).

A connection between the biosynthesis of thiamine and that of pantothenate has been previously noted (8, 21, 42). The finding that exogenous pantothenate replaced the requirement for thiamine in a number of mutant strains of *S. enterica* serovar Typhimurium (9, 26) reemphasized the need to understand the connection between these two biosynthetic pathways. For instance, strains lacking the first enzyme in the common pathway to purines and the pyrimidine moiety of thiamine, PurF,

are thiamine auxotrophs under some conditions, most notably when glucose is the sole carbon source (9, 26). The presence of exogenous pantothenate, resulting in elevated CoA levels, eliminated the requirement for thiamine in these strains (9, 11, 25). In addition, mutants with lowered levels of CoA have a thiamine requirement when flux through the purine biosynthetic pathway is reduced (11).

Mutations that suppressed the thiamine requirement of a *purF* mutant and resulted in increased accumulation of pantothenate in the growth medium have been isolated (9). It is shown here that the causative mutation in these strains is the insertion of one CG base pair upstream of the transcription start site of the *panBCD* operon. This base addition between the -10 and -35 hexamers of the promoter results in a 10-fold increase in transcription of the *pan* genes. It is further shown that increased expression of *panB* alone is sufficient to increase pantothenate levels and restore thiamine synthesis in a *purF* mutant.

Suppression of the thiamine requirement is caused by increased pantothenate biosynthesis. Frequently arising (10^{-5}) mutations that cause pantothenate excretion and allow *purF* or *purH* mutants to grow in the absence of thiamine have been described (9). The causative lesions in these strains were found to be closely linked, by P22 transduction, to the *panBCD* genes at 3 min on the *S. enterica* serovar Typhimurium chromosome (9). Three phenotypes associated with these mutations were consistent with increased synthesis of pantothenate in these strains. First, strains containing the relevant mutation accumulated ~ 10 -fold more pantothenate in the growth medium than did an isogenic wild-type strain, as quantified by a bioassay (data not shown) (9). Second, these strains were impaired for uptake of exogenous pantothenate, as a consequence of increased internal pools of pantothenate (31, 37). When pantothenate uptake assays (36) were performed with relevant mutant strains (Fig. 2), strain DM1632 (*zae-3653::Tn10 panBp654*) had a decreased ability to take up pantothenate from the medium

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FIG. 1. Pantothenate and CoA biosynthetic pathways. (A) Pantothenate biosynthetic pathway in *S. enterica* serovar Typhimurium. (B) CoA biosynthetic pathway in *E. coli*. Where known, the genes encoding the products catalyzing each step are indicated.

compared to that of wild-type strain DM1633 (*zae-3653::Tn10*). The defect measured in the *panBp654* mutant was quantitatively similar to that of mutants either lacking the pantothenate permease (*panF*) (17, 38) or partially defective in the

FIG. 2. Uptake of exogenous pantothenate is reduced in strains containing *panF*, *coaA_{ts}*, or *panBp654* mutations. Strains were grown in rich nutrient broth (NB) medium at 30°C overnight, and pantothenate uptake assays were performed as described by Vallari and Rock (38). [^{14}C]pantothenic acid (sodium salt) with a specific activity of 51.5 mCi/mmol was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, Mo.) and used at a final concentration of 1 μM . The strains tested were DM5126 (*panB629 zxx-3124::Tn10*) (■), DM5125 (*panB629 zxx-3124::Tn10 panF671*) (□), DM1632 (*zae-3653::Tn10 panBp654*) (●), DM1633 (*zae-3653::Tn10*) (○), and DM5133 (*pan-629 coaA_{ts}*) (▲). Multiple experiments were performed with similar results, and data from one such experiment are shown.

essential pantothenate kinase CoaA (*coaA_{ts}*) (11, 37), i.e., DM5125 and DM5133, respectively. We suggest that the decreased uptake in the *panBp654* mutant is mechanistically similar to that of a *coaA_{ts}* mutant; that is, internal levels of pantothenate sufficient to inhibit the PanF permease have accumulated. It was unlikely that the causative mutations affected *panF* or *coaA* directly, since plasmids carrying *panF* or *coaA* did not alter the pantothenate uptake profile of the defective strain (data not shown). Finally, the mutant strain carrying *panBp654* (DM1632) had 50% more CoA than did the isogenic wild-type strain (0.73 ± 0.06 versus 0.52 ± 0.04 nmol/mg of dry weight, respectively). While this is not a large difference, this result is consistent with previous reports that elevated pantothenate levels increase CoA levels a maximum of twofold (7, 31).

The *panBp654* mutation results in increased transcription of the *panBCD* operon. Previous work suggested that *panBCD* is an operon (7, 20). Analyses of strains carrying a transcriptional fusion in *panC* were consistent with this hypothesis. In particular, the presence of a *panB614::Tn10d(Tc)* insertion reduced transcription from *panC607::MudJ* 10-fold, indicating that at least *panB* and *panC* were transcribed by the same promoter. These data, shown in Table 1, justified the use of *panC607::MudJ* to monitor transcription of the *panBCD* operon.

To determine if the *panBp654* mutation mediates pantothenate overproduction simply by increasing transcription of the *panBCD* operon, an isogenic pair of strains were constructed and transcription through the operon was monitored. Data from these experiments (Table 1) showed that the *panBp654*

TABLE 1. *panBp654* increases transcription of the *panBCD* operon^a

Strain	Relevant genotype	β -Galactosidase activity (Miller units) ^b
DM309	<i>panC607::MudJ</i> ^c	36 $\pm$ 2
DM308	<i>panC607::MudJ panB614::Tn10d(Tc)</i> ^d	3.5 $\pm$ 0.1
DM6097	<i>panC607::MudJ zae-3653::Tn10</i>	37.1 $\pm$ 2
DM6098	<i>panC607::MudJ zae-3653::Tn10 panBp654</i>	358 $\pm$ 28

^a Cells from a full-density NB culture were inoculated into NB and grown to an optical density at 650 nm of ~ 0.3 . β -Galactosidase assays were performed as previously described (10, 23). All strains are derivatives of LT2 and were generated for this study or were part of the laboratory collection. The locations of insertions in *pan* genes were determined by nutritional studies and confirmed by sequence analyses.

^b A Miller unit is defined as 1 nmol of *o*-nitrophenyl- β -D-galactopyranoside hydrolyzed per min. Each reported value is the average of at least three independently grown cultures assayed in duplicate along with the standard deviation.

^c MudJ is used throughout to refer to the Mu dI 1734 transposon described previously (5).

^d Tn10d(Tc) is used throughout to refer to the transposition-defective mini-Tn10 (Tn10 Δ 16 Δ 17) described by Way et al. (41).

mutation caused an approximately 10-fold increase in *panC* transcription. This increase was qualitatively consistent with the increased excretion of pantothenate observed in the *panBp654* mutant and suggested that the *panBp654* lesion was upstream of the *panBCD* promoter.

The *panBp654* mutation optimizes the *panBCD* promoter.

To identify the *panBp654* lesion, a DNA fragment from strain DM1632 (*panBp654*) that conferred reduced pantothenate uptake when present on a plasmid was identified. Two plasmid clones (pAR2 and pAR3) that decreased pantothenate uptake were identified, and their effect on uptake is shown in Fig. 3A. While these plasmids caused a dramatic decrease in pantothenate uptake in the *panB* mutant strain, this was primarily due to complementation of the *panB* mutant restoring prototrophy to the strain (see below). However, these two plasmids (pAR2 and pAR3) resulted in a slight (about twofold) but reproducible decrease in pantothenate uptake by the *panB* mutant strain compared to a plasmid containing the wild-type *panBCD* operon (data not shown). Based on this difference, we concluded that the region of the DNA carrying *panBp654* was present on the plasmid. The region contained in one of these plasmids was amplified from the *panBp654* mutant and wild-type strains by PCR and ligated into the *Hinc*II site of a low-copy vector, pMAK705 (14), generating pAR4 and pAR5, respectively. These plasmids were moved into wild-type strain LT2 by electroporation. Sequence analysis confirmed that the orientation of the insert was the same in both plasmids. A low-copy vector was used since data suggested that copy number was capable of partially masking the relevant phenotype. The resulting strains were assessed for pantothenate uptake, and the data are shown in Fig. 3B. The strain carrying a plasmid derived from the *panBp654* mutant strain (pAR4) had

FIG. 3. Pantothenate uptake assays define the locus affected by *panBp654*. (A) Relevant strains were grown in rich medium overnight at 30°C. For strains carrying plasmids, the medium was supplemented with chloramphenicol at 20 μ g/ml. Pantothenate uptake assays were performed as described in the legend to Fig. 2. The strains assayed were DM97* (■), DM97/pAR2 (○), and DM97/pAR3 (△). (B) Similar analyses were performed with strains DM97/pAR5 (□), DM97/pAR4 (■), and DM97/pAR6 (●). For both panels A and B, multiple experiments were performed with similar results and data from a representative experiment are shown. (C) Polyclonal antibodies (anti-rabbit immunoglobulin G) against PanB were generated by the animal care facility at the University of Wisconsin—Madison Medical School. Western blot analysis was performed as described by Harlowe and Lane (15). Equal amounts of protein were loaded in three lanes containing DM97 carrying pAR5, pAR4, or pAR6 as indicated. Strain DM97* contains a Tn10d(Tc) insertion in *panB* [*panB611::Tn10d(Tc)*]. The growth requirements of this strain are satisfied by pantoate alone, suggesting that the promoter in the Tn 10 element directs the transcription of the downstream *panCD* genes (6).

FIG. 4. The *panBp654* mutation increases the spacing between promoter hexamers. (A) RNA was isolated from mid-logarithmic-phase cells with a Qiagen RNeasy kit (Qiagen, Chatsworth, Calif.) and quantitated by monitoring A_{260}/A_{280} . The primer (5' GGTAATGTCGC GAAGCGTTT 3') was end labeled with [γ - 32 P]ATP (~7,000 Ci/mmol; ICN, Irvine, Calif.) with T4 polynucleotide kinase (Epicentre, Madison, Wis.) for 30 min at 37°C, followed by a 5-min incubation at 70°C to inactivate the enzyme. A DNA ladder used to map the transcription start site was generated with pWT48-1 as the template by using a SequiTherm Excel II sequencing kit (Epicentre). The labeled primer was also used with 50 μ g of total RNA isolated from strains DM1632 (*panBp654 zae-3653::Tn10*) and DM1633 (*zae-3653::Tn10*) and extended with Life Technologies (Rockville, Md.) ThermoScript RT enzyme for 1 h at 65°C. The products were separated on a 6% denaturing acrylamide gel, visualized, and quantified via Phosphor-Imager (Molecular Dynamics, Piscataway, N.J.). (B) Schematic representation of the *panBCD* promoter region. Computer-assigned promoter hexamers are underlined. The solid arrow indicates the run of C's, of which there are six in the wild-type strain and seven in the *panBp654* mutant strain. Transcription start sites were identified by primer extension and are indicated by open arrows. Lowercase letters indicate the start of the PanB coding sequence.

a lower rate of pantothenate uptake than the strain carrying a plasmid with DNA amplified from the wild type (pAR5), confirming that the *panBp654* mutation was contained in the insert DNA.

Complete sequence analysis was performed on pAR5 (1.45-kb fragment of wild-type origin) and pAR4 (1.45-kb fragment of

TABLE 2. Multicopy *panB* is sufficient to increase CoA levels in *S. enterica* Serovar Typhimurium

Strain	Relevant genotype	CoA level (nmol/mg of dry wt) ^a
DM2047	<i>purF2085</i> /pSU19	0.63 $\pm$ 0.09
DM5687	<i>purF2085</i> /ppanB	1.00 $\pm$ 0.17
DM5689	<i>purF2085</i> /ppanC	0.75 $\pm$ 0.16
DM5690	<i>purF2085</i> /ppanD	0.64 $\pm$ 0.11

^a CoA levels were determined by the method of Allred and Guy (2). The levels shown are averages of three cultures from independent colonies.

panBp654 origin) at the University of Wisconsin—Madison Biotechnology Sequencing Center. Sequences were generated from at least five different primers for a total of 20 reactions spanning the entire insert. Sequence analysis of these clones determined that (i) the only complete ORF encoded by the insert DNA was *panB* and (ii) a single base pair insertion upstream of the *panB* gene distinguished the *panBp654* allele from the wild-type DNA.

To determine if the single C base insertion into the coding strand 5' of *panB* is sufficient to cause the phenotypes associated with *panBp654*, this mutation was incorporated by site-directed mutagenesis and confirmed by sequence analysis, generating pAR6. Pantothenate uptake assays (Fig. 3B) confirmed that the single base insertion in pAR6 was sufficient to impair pantothenate uptake. Consistently, both pAR4 and pAR6 increased PanB levels in the strain compared to pAR5, as measured by Western analysis (Fig. 3C).

Sequence alignment programs were used to predict the -10 and -35 hexamers of a promoter upstream of the *panB* coding sequence. The sequence upstream of *panB* is shown in Fig. 4B, and the putative promoter element is indicated. Primer extension analyses showed that the transcription start site was the same in both the mutant and wild-type strains (Fig. 4A) and consistent with the promoter assignment shown. The addition of the base (C) defining the *panBp654* allele increased the spacing between the promoter hexamers from 16 to 17 bp, which is considered to be optimal (3, 4, 27). These data together allowed us to conclude that the *panBp654* mutation generates a promoter nearer the consensus and results in increased transcription of the *panBCD* operon.

Mutations causing pantothenate excretion are the most frequently occurring suppressors of strains compromised for synthesis of the hydroxymethyl pyrimidine moiety of thiamine in *S. enterica* serovar Typhimurium (e.g., *purF* mutants on glucose medium containing adenine). The molecular description of the *panBp654* mutation provided a means by which to explain this frequency, which is higher than expected for "promoter-up" mutations. The addition of a base in a run of similar bases, such as that described in a run of six C's, can occur by the slipping of DNA polymerase (33, 39, 40). Three independently isolated mutations that caused the phenotype described here were the result of an additional base in this run of C's. This result suggests that, while not the only one, this mutation is the most frequent way to increase the synthesis of pantothenate in *S. enterica* serovar Typhimurium. The ease with which the strength of the *pan* promoter can be enhanced 10-fold suggests that the lower wild-type level of expression has been maintained for metabolic efficiency, since the regulation of CoA is

FIG. 5. *panB* in multicopy allows PurF-independent thiamine synthesis. Growth curve analyses were performed as previously described (25). The ability of strain DM1936 (*purF2085*) to grow in the absence of thiamine was monitored with each of four plasmids present in the strain. The medium used was minimal glucose medium containing either adenine (0.4 mM) (open symbols) or adenine and thiamine (100 nM) (closed symbols) at 37°C. The strains used were DM1936/pSU19 (○, ●), DM1936/ppanB (□, ■), DM1936/ppanC (△, ▲), and DM1936/ppanD (◇, ◆).

such that the potential benefit of increased transcription would be negated by loss of pantothenate to the medium.

Increased PanB is sufficient to allow overproduction of pantothenate and result in a PanP phenotype. The minimal clones that increased pantothenate synthesis contained not only the mutation in the promoter described above but the complete *panB* gene. This observation suggested that while the *panBp654* mutation resulted in increased transcription of the operon, overexpression of PanB was sufficient for the resulting phenotypes. To address this possibility, single-gene clones of *panB*, *panC*, and *panD* were constructed by PCR amplification of the relevant products from a wild-type strain. In each case, the resulting plasmids complemented strains defective in the respective *pan* gene and the *lac* promoter encoded in the host plasmid (pSU19) was responsible for transcribing the *pan* gene. Total CoA levels were measured in *purF* strains harboring each of the three clones, and the results are shown in Table 2. While a dramatic effect on CoA levels was not observed, the plasmid containing *panB* resulted in a slight (50%) but reproducible increase in the CoA level. Significantly, in a physiologically relevant *in vivo* assay, the *panB* clone restored thiamine-independent growth of a *purF* mutant on glucose adenine medium while the other two clones did not (Fig. 5). Taken together, the data in Table 2 and Fig. 5 emphasize the significance that minor changes in metabolite pools can have for the physiology of the cell.

It is worth noting that there are reports in the literature suggesting either PanC (7) or PanD (19) is the limiting step in pantothenate synthesis, while our data suggest that PanB has this role. The fact that the strains used in this study were wild type for the pantothenate biosynthetic genes may be a significant difference between these reports.

Conclusion. From the data herein, we concluded that the *panBp654* mutation results in increased transcription of the *pan* operon and that the increased transcription of *panB* was, in turn, sufficient to allow increased pantothenate synthesis. We hypothesize that the slight increase in CoA levels caused by this increased internal pantothenate synthesis is the metabolic consequence that allowed PurF-independent thiamine synthesis. The specific requirement for CoA in the efficient synthesis of thiamine remains unclear and is under investigation.

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