**thiBPQ Encodes an ABC Transporter Required for Transport of Thiamine and Thiamine Pyrophosphate in Salmonella typhimurium**

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In *Salmonella typhimurium*, thiamine pyrophosphate (TPP) is a required cofactor for several enzymes in central metabolism. Herein we identify a new *thi* operon, *thiBPQ* (designated *sfaABC* in *Escherichia coli*), required for the transport of thiamine and TPP into the cell. Insertions in the operon result in strains that are phenotypically and biochemically defective in thiamine and TPP transport. Data presented herein show that this operon is transcriptionally repressed in the presence of exogenous thiamine, with TPP the likely regulatory molecule. This work represents the first identification of thiamine transport genes in bacteria and demonstrates the function of a proposed ABC transporter in *E. coli*.

Thiamine pyrophosphate (TPP)[1] is a required cofactor synthesized *de novo* in *Salmonella typhimurium*. The primary role for TPP is in central metabolism as an electron carrier and nucleophile for such enzymes as pyruvate dehydrogenase (EC 1.2.4.1), acetylacetate synthase (EC 4.1.3.18), and α-ketoglutarate dehydrogenase (EC 1.2.4.2). Despite its importance in cellular physiology, neither the *de novo* biosynthetic pathway nor the salvage systems for thiamine are fully understood in any organism.

Thiamine monophosphate (TMP) is generated by the condensation of two independently synthesized moieties: 4-amino-5-hydroxy-methyl pyrimidine pyrophosphate (HMP-PP) and 4-methyl-5-(β-hydroxyethyl) thiazole phosphate (THZ-P). TMP is then phosphorylated by the action of thiamine monophosphate kinase, ThiL (1), to form the physiologically relevant form of the vitamin, TPP. Recently several studies in the enteric bacteria *S. typhimurium* and *Escherichia coli* have elucidated many steps in the formation of thiamine (2–5), but much of the pathway remains unknown.

Mutants defective in various steps in *de novo* synthesis can be supplemented exogenously with THZ, HMP, thiamine, TMP, or TPP. These results suggested that *S. typhimurium* had the ability to take up and incorporate these compounds into the *de novo* thiamine biosynthetic pathway. It was demonstrated several years ago that thiamine was actively transported in *E. coli*, and this transport was shown to involve a thiamine-binding protein whose activity was repressed by excess thiamine (6–9).

The transport of TPP was not addressed in these previous studies. The presence of the thiamine-binding protein led to the hypothesis that thiamine was transported via a periplasmic binding protein-dependent ABC-type transporter (10).

We report here the identification of an operon (*thiBPQ*) at centisome (Cs) 1.5 on the *S. typhimurium* and *E. coli* chromosomes involved in the specific translocation of thiamine and its phosphoesters across the inner membrane. Analysis of the *E. coli* sequence (designated *sfaABC*) in addition to phenotypic analysis in *S. typhimurium* suggested that *thiBPQ* encoded thiamine binding protein, inner membrane channel, and energy-transducing ATPase, respectively. Transcriptional fusions in this operon were regulated in response to exogenous thiamine.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains**

All strains used in this study are derivatives of *S. typhimurium* LT2 and are listed in Table I. MudJ is used throughout the paper to refer to the MudI 1374 transposon, which has been described (11), and Tn10d(Tc) refers to the transposition defective mini-Tn10 (Tn10ΔMudJ) (12).

**Culture Media and Biochemicals**

No-carbon source E media (NCE) supplemented with 1 mM MgSO₄ and 11 mM glucose was used as minimal media (13, 14). Difco nutrient broth (NB, 8 g/liter) with NaCl (5 g/liter) added was used as rich medium. Difco BiTek agar (15 g/liter) was added for solid medium. Antibiotics were added as needed to the following concentrations in rich and minimal media respectively: kanamycin (50, 125 μg/ml), tetracycline (20, 10 μg/ml), and chloramphenicol (40, 4 μg/ml). Radiolabeled thiamine (C2-14C-THZ-thiamine) with a specific activity of 24 mCi/mmol was purchased from Amersham Pharmacia Biotech (Arlington Heights, IL). All other chemicals were purchased from Sigma.

**Genetic Methods**

**Transduction Methods**—All transductional crosses were performed by using the high frequency transducing bacteriophage P22 mutant HT 105/1 int-201 (15) as described (16). Transductants were purified and identified as phage-free by cross-streaking on green plates (17).

**Mutant Isolation**—Strains defective for TPP transport were isolated by insertional mutagenesis with one of two transposons, Tn10d(Tc) or MudJ. To facilitate mutant isolation, a pool of cells containing >80,000 independent insertions was generated as described elsewhere (18, 19). A P22 lysate was grown on these cells to generate either a MudJ or Tn10d(Tc) phage pool.

To isolate Tn10d(Tc) insertion mutants, the Tn10d(Tc) phage pool described above was used to transduce a strain defective in *de novo* thiamine synthesis (either DM62 (thi-924::MudJ) or DM460 (thiH910::MudJ)) to tetracycline resistance (Tc⁺) on NB-tetracycline plates. The Tc⁺ transductants were screened for those that were able to grow with 1 μM thiamine but not 1 μM TPP. Putative insertion mutants defective in high affinity TPP transport (defining the *thiP* locus) were streaked for phage sensitivity and saved for further analysis.

Point mutations defective in high affinity TPP transport were isolated as described above with the following exception. The Tn10d(Tc) pool utilized had been mutagenized with hydroxylamine as described (13, 20), resulting in the isolation of point mutations linked to a Tn10d(Tc) element.

After mapping, MudJ insertions in the *thiP* locus were identified.
The protocol for the uptake assay used for thiamine and TPP was a combination of previously described methods (7, 29) and is summarized below. Overnight cultures grown in NB were pelleted and resuspended in an equal volume of 0.85 mM NaCl. 0.5 ml of resuspended cells were inoculated into 10 ml of minimal medium and incubated with shaking at 37 °C until the optical density at 560 nm was ~0.4. Cultures were then pelleted, resuspended in 2 ml of minimal medium, separated into 1-ml aliquots, and stored on ice until needed. The cultures were equilibrated at 37 °C for 10 min, and assays were initiated by the addition of a radioactive substrate (final concentration of 230 nM for [32P]TPP or 460 nM [14C]-labeled thiamine) to 1 ml of cells. Fractions (0.2 ml) were removed at specific time points, rapidly filtered through HA-type Millipore 0.45-μm nitrocellulose filters (Bedford, MA), and washed with 20 ml of NCE salts medium. Filters were dried under a 150 watt lamp, mixed with 5 ml of scintillation fluid, and counted (1 min for [32P]TPP and 2 min for [14C]-thiamine) in a Packard Instruments Model 4530 Scintillation Counter. 

Growth Curves

Curves were done aerobically as described (16). Final concentrations of THZ, thiamine, TMP, and TPP were as indicated. 

Generation of [β-32P]TPP

[32P]TPP was generated using cell-free extracts of a strain overproducing ThiL as described (1) with the following exceptions. The ThiL reaction was initiated with the addition of 15 μl of ATP (10 μl of 100 mM ATP + MgCl2 and 5 μl of γ-[32P]ATP (specific activity 6000 Ci/mmol)). Radiolabeled [β-32P]TPP was purified from the ThiL reaction mix via column chromatography as described by Matsuda and Cooper (27) with the following exceptions. Twenty fractions (3 ml each) from the 60 ml of 0.1 mM buffer (pH 3.5) were collected. The TPP elution profile was tested by bioautography with strain DM1683 (thiI933:10d(Tc)) which qualitatively determined the fractions containing significant TPP. To determine the radiochemical purity and concentration of the TPP, high pressure liquid chromatography analysis was performed on an aliquot of this fraction, as described previously (1, 28). The high pressure liquid chromatography fraction containing the TPP peak was collected and scintillation counted for 1 min in a Packard Instruments Model 4530 Scintillation Counter (Downers Grove, IL), indicating that the TPP accounted for ~80% of the label. The specific activity of the TPP was calculated to be 9400 Ci/mmol.

Uptake of Radiolabeled TPP and Thiamine

Strain Genotype

| Strain | Genotype |
|--------|----------|
| LT2    | Wild type |
| BL2/1/DE3 | hsdS gal (0)S8857 ind1 Sam7 nin5 lacUV5-T7 gene 1 (E. coli) |
| DM62   | thi-924::MudJ |
| DM460  | thi-910::MudJ |
| DM1683 | thi-935::10d (Tc) |
| DM688  | thi-935::10d (Tc; thi-995:10d::MudJ) |
| DM2275 | thi-942::10d (Tc) |
| DM2572 | BL2/1/DE3/3/Thi-6 |
| DM3340 | thi-935::10d (Tc; thi-995:10d::MudJ) |
| DM3403 | zac-8602::MudJ thi-995:10d::MudJ |
| DM3408-3411 | zac-8603::10d (Tc) thi-9104::10d::MudJ |
| DM3412 | zac-8603::10d (Tc) thi-9104::10d::MudJ |
| DM3413 | leu-485::thi-942::10d (Tc) |
| DM3616 | leu-485::thi-942::10d (Tc) |
| DM3617-3656 | thi-1027-1066::MudJ thi-942::10d (Tc) |
| DM3670 | zac-8048::10d (Tc) thi-9279::10d::MudJ |
| DM3671 | zac-8048::10d (Tc) thi-9279::10d::MudJ |
| DM3781 | zac-8048::10d (Tc) thi-91054::MudJ |
| DM3782 | thi-1026::MudJ |
| DM3922 | thi-1024::MudJ |
| DM3924 | thi-1024::MudJ |
| DM3925 | thi-1024::MudJ thi-1012::10d (Tc) |
| DM3930 | thi-10154::MudJ |
| DM3931 | thi-10154::MudJ thi-1012::10d (Tc) |

a MudJ is used throughout the text to refer to the Mud dl1734 transposon (11).

b ThiL insertion referred to the transposition-defective mini-Tn10 (Thi-995 Δ) (12).

Using co-transduction with the leu locus in strain DM3616 (Δleu-485::thi-942::10d(Tc)) and the MudJ pool described above. Isolation of MudJ insertions linked to thiP locus Thi-104d(Tc) in strain DM3340 (thi-935::10d; thi-995:10d::MudJ) utilized a positive screen, as described (19). One highly linked insertion, DM3403 (zac-8602::MudJ thi-995:10d Δ) (97%), was saved for further analysis.

Molecular Biology Techniques

DNA Sequencing—DNA was sequenced at the University of Wisconsin-Madison Biotechnology Center-Nucleic Acid and Protein Facility. DNA sequence analysis program BLAST (21) was used to compare this sequence with known sequences from the data base.

Chromosome Location of thiP Operon—The TPP transport-deficient mutations were mapped on the S. typhimurium chromosome via sequencing MudQ phage DNA from strain DSM468 (zac-8602::MudQ thi-995:10d Δ) which had been generated from strain DSM403 (zac-8602::MudJ thi-995:10d Δ) as described (22). The resulting locked-in P22 phage was induced, and DNA was isolated as described (23). The purified DNA was then used as a template for DNA cycle sequencing using a Sequitherm (Epicentre Madison, WI) kit. The primer used was Mura (5'-GAAAAGGTTCCGTTGTTGCA-G-3') which hybridizes to the right end of the MudQ insertion.

Mapping of Insertions by PCR—The location of four insertions in the thiP operon were determined via a PCR-based protocol (24). Amplification between the insertions was done using Vent (exo-) polymerase (New England Biolabs, Inc., Beverly, MA) in a Thermolyne Temp-Tronic Thermocycler (Dubuque, IA). Reaction conditions were as follows: 95 °C denaturation for 1 min, 55 °C annealing for 1 min, and 72 °C extension for 2 min. Primers used were: Tn10-1 (5'-GACAAGATGTTATCCACCTAAAC-3'), which hybridizes to the 66-base pair inverted repeat Tn10 sequence; Mula (5'-ATCCGGATAATCTTGGGCGC-3'), which hybridizes to the left end of the MudJ insertion; and Mura (defined above). Additional MgSO4 was added to all reaction mixes to a final concentration of 1 mM. Amplified products were visualized via agarose gel electrophoresis, purified using Qiagel gel extraction kit (Qiagen, Chatsworth, CA), and sequenced at the University of Wisconsin-Madison Biotechnology Center-Nucleic Acid and Protein Facility.

β-Galactosidase Assays

Assays were performed using the Miller method (25) as described previously (26).

RESULTS

Isolation of TPP Transport Defective Mutants—Forty-seven independently isolated mutations causing a similar thi phenotype were identified, including 2 point mutations, 5 Thi-10d, and 40 MudJ insertions. Phase P22 co-transduction analysis genetically mapped all of the insertions to the same locus, designated thiP (>90% linked). Growth curve analysis, represented in Fig. 1, revealed two significant points. Double mutants defective in both de novo synthesis (thiH) and the thiP locus required 1000-fold more TPP (100 nM versus 100 μM) for maximal growth than the single thiH mutant (Fig. 1, B and D), and yet these strains could reach optimal growth rates when supplied with >1 μM exogenous thiamine (Fig. 1, A and C). Additionally, thiP mutations in a wild-type background had no observable growth defects in minimal medium.

Physical Mapping of Insertions—The location of the thiP locus on the S. typhimurium chromosome was determined by sequencing the flanking DNA of a MudQ insertion (zac-8602::MudQ) known to be linked to thiP. BLAST (21) computer data base analysis determined that the insertion was 2° to the end of araC at Cs 1.5 based on sequence similarity to the E. coli genome sequence. Conclusively, all of the mutations with P22 determined that thiP was >95% linked to the thiamine biosynthetic operon, confirming that thiP resided at Cs 1.5 in S. typhimurium and that the gene order was consistent with the predicted order from the E. coli chromosome.

Four insertions in the thiP locus were physically mapped with a PCR-based protocol. Primers designed to the ends of the insertions were used to amplify DNA in strains containing both
a MudJ and Tn10(d) insertion in the thiP locus. Amplified products (~400 base pairs) from strains DM3925 and DM3931 were purified and sequenced. Comparison with the E. coli data base using BLASTN determined that the two insertions in strain DM3925 were in sfuA. S. typhimurium amino acid sequences are depicted, residues conserved with E. coli are in bold. We designate these genes thiBPQ to more clearly define involvement in thiamine salvage. As shown, thiBPQ maps to Cs 1.5 on the S. typhimurium and E. coli chromosomes in between the leucine biosynthetic genes and arabinose utilization.

FIG. 1. Thiamine and TPP requirement of thiH and thiPthiH mutants. To determine the phenotypes of a thiPthiH double mutant relative to a thiH mutant, growth curves were performed on strains of DM2275 (thiH942::Tn10(Tc)) (A and B), and DM3652 (thiB1062::MudJ thiH942::Tn10(Tc)) (C and D) in minimal medium supplying varying concentrations of thiamine and TPP. Symbols represent the amounts of thiamine (A and C) or TPP (B and D) supplemented: ■, 1 mM; △, 100 μM; ▽, 10 μM; ●, 1 μM; ○, 100 nM; and □, no supplement.

FIG. 2. Relative location of four insertions in the thiP locus. Four insertions in the thiP locus were physically mapped via a PCR-based protocol. Sequence comparison to E. coli determined that the two insertions in strain DM3925 were in sfuA, whereas the insertions in strain DM3931 were in sfuC. S. typhimurium amino acid sequences are depicted, residues conserved with E. coli are in bold. We designate these genes thiBPQ to more clearly define involvement in thiamine salvage. As shown, thiBPQ maps to Cs 1.5 on the S. typhimurium and E. coli chromosomes in between the leucine biosynthetic genes and arabinose utilization.
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These data clearly showed that the insertions in ability to transport radiolabeled thiamine and TPP (Fig. 3). Porters, mutants defective in the operon were tested for their test whether the thiBPQ [32P]TPP (assays were performed using both radiolabeled 14C-thiamine (protein motif and one nucleotide binding site, respectively. Determined that ThiB and ThiQ contained a bacterial binding components using the PROSITE data base (30). These analyses determined that the operon encoded the predicted trans- a defect in the transport of both thiamine and TPP. LT2 yielded in GenBankTM. Were only present in proteins that had significant homology with all ABC transporters, ThiB was used as a marker. BLASTP (21) analysis with the E. coli proteins determined that all three components of the transport complex were only present in Hemophilus influenza. No other significant homologs for ThiB were found in any other organism listed in GenBankTM.

Motif searches were also performed on the complex components using the PROSITE data base (30). These analyses determined that ThiB and ThiQ contained a bacterial binding protein motif and one nucleotide binding site, respectively.

Mutations in thiBPQ Are Defective in TPP Transport—To test whether the thiBPQ operon encoded the predicted transporter, mutants defective in the operon were tested for their ability to transport radiolabeled thiamine and TPP (Fig. 3). These data clearly showed that the insertions in thiBPQ caused a defect in the transport of both thiamine and TPP. LT2 yielded rates of uptake of 2.9 ± 0.07 pmol of TPP/min/A860 nm and 6.3 ± 0.34 pmol of thiamine/min/A560 nm, whereas insertions in thiB or thiQ had rates that were ≤ 0.

As stated earlier, mutants blocked in both de novo synthesis and thiBPQ were able to grow in the presence of 1 μM thiamine but not TPP. This result suggested there was an additional mechanism for transport of thiamine that was independent of ThiBPQ. To confirm that thiamine accumulated in a thiB mutant under these conditions, uptake assays were performed with 460 nM, 4.6 μM, and 23 μM thiamine (data not shown). Slight rate increases in accumulation of thiamine could be seen (no transport at 460 nM to 2.03 ± 0.32 pmol of thiamine/min/A560 nm at 23 μM).

thiBPQ Comprise a TPP-regulated Operon—Although an operon structure was expected based on the E. coli sequence, a strain containing a MudJ transcriptional fusion in thiQ was used to confirm this assumption. Strains DM3926 (thiQ1054::MudJ) and DM3931 (thiQ1054::MudJ thiB1012::Tn10d(Tc)) produced ~30 and ~4 units of β-galactosidase activity, respectively, when grown in minimal medium, consistent with an operon structure for the thiBPQ gene cluster.

Data presented in Table II demonstrate that thiBPQ belonged to the growing number of TPP transcriptionally regulated genes in S. typhimurium (4, 31). This experiment was complicated by the fact that the transcriptional fusions in the genes of interest were defective for the transport of thiamine and TPP. We were able to circumvent this problem since we had determined that thiamine could accumulate in a thiB mutant when provided at concentrations >1 μM. As shown in Table II, β-galactosidase activities varying from 70 to 20 units were obtained in minimal medium using different insertions in the operon; however, in the presence of exogenous thiamine (1 mM), expression was reduced 3–4-fold (Strains DM3781 and DM3782). To address whether this response was to thiamine or TPP, the previously characterized thiL927 point mutation was utilized (1). Strains containing this point mutation and a thi reporter are completely repressed by TPP, but not thiamine due to altered thiamine monophosphate kinase, ThiL (EC 2.7.4.16), activity. Two insertions thiB1062::MudJ and thiQ1054::MudJ were transduced into a thiL927 point mutation background, generating strains DM3671 and 3670, respectively. As shown in Table II, strains containing the thiL927 point mutation were not repressed completely in response to thiamine, while the isogenic strain exhibited normal repression. These data are consistent with repression of the thiBPQ operon occurring in response to TPP, as are other previously described thiamine biosynthetic operons.

**DISCUSSION**

Work presented here identifies a new ABC transporter (thiBPQ) in S. typhimurium. We show here that the TPP-regulated thiBPQ operon at C.t 1.5 is responsible not only for the transport of thiamine but also TPP in S. typhimurium. This agrees with previous work in E. coli which demonstrated that the thiamine transport was energy-dependent, independent of de novo biosynthesis, and repressed by thiamine (possibly TPP) (8, 32). More recently, it was shown in E. coli that the ThiB protein has high affinity for the binding of thiamine, TMP, and

**Table II**

| Strain | Genotype | β-Galactosidase activity |
|--------|----------|-------------------------|
|        |          | Minimal                  | Minimal 1 μM thiamine |
| DM3670 | thiQ1054::MudJ zaj-8048::Tn10d(Tc) thiL927 | 31.9 ± 5.1 | 19.3 ± 4.0 |
| DM3781 | thiQ1054::MudJ zaj-8048::Tn10d(Tc) | 20.6 ± 0.6 | 5.9 ± 1.1 |
| DM3671 | thiB1062::MudJ zaj-8048::Tn10d(Tc) thiL927 | 80.0 ± 7.2 | 55.5 ± 4.1 |
| DM3782 | thiB1062::MudJ zaj-8048::Tn10d(Tc) | 70.0 ± 7.4 | 25.6 ± 3.2 |

**Fig. 3.** ThiBPQ mediates thiamine and TPP uptake. Uptake assays were performed using both radiolabeled 14C-thiamine (A) and [32P]TPP (B) as described under “Experimental Procedures.” In panel A, thiamine uptake was tested in an LT2 (●) and DM3922 (●) (thiB1062::MudJ) background. Data, including standard deviations for three independently performed thiamine uptake assays are shown. In panel B, TPP transport was tested in LT2 (●), DM3922 (●), and DM3926 (●) (thiQ1054::MudJ). Representative data from more than three independently performed TPP uptake assays are shown.
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TPP, suggesting that *thi*BPQ is also responsible for TMP transport. This three-gene operon is present in *E. coli* at the same chromosome location; therefore, we propose that the gene designations be changed from *sfuABC* to *thi*BPQ to more clearly define the role of this operon in thiamine metabolism.

A recent publication (33) has identified the thiamine transport gene in *Saccharomyces cerevisiae*. Unlike the transport system for thiamine and TPP in *E. coli* and *S. typhimurium* (ABC transporter), this gene is a member of the major facilitator superfamily (MFS) of transporters (34). The MFS class of transporters differs from ABC-type transporters in two significant ways: the translocation complex is encoded by a single gene, and the energy for translocation does not come from ATP hydrolysis but is carrier mediated. *S. cerevisiae* is unable to transport TPP (35). It is interesting that a different mechanism for thiamine transport has evolved and that these two systems differ not only in structure but in substrate recognition.

The fact that strains containing mutations in *thi*BPQ and a *de novo* block in thiamine biosynthesis were corrected with 100-fold less thiamine (1 μM) than TPP (100 μM) suggested that there was another mechanism for thiamine uptake in *S. typhimurium*. In fact we could show that, at concentrations >4.6 μM, thiamine did accumulate in a *thi* mutant. We propose that this accumulation was due to a low affinity thiamine transport. This three-gene operon is present in *E. coli* (36). Analysis of the *thi* box in 5′ to translation in all genes found to be regulated by TPP predicts the existence of a TPP-responsive regulatory protein.

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