Characterization of *thiI*, a New Gene Involved in Thiazole Biosynthesis in *Salmonella typhimurium*

ERIC WEBB, KATHY CLAAS, AND DIANA M. DOWNS*

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

Received 13 January 1997/Accepted 13 April 1997

Thiamine pyrophosphate (TPP) is a required cofactor in *Salmonella typhimurium* that is generated de novo by the condensation of 4-amino-5-hydroxymethyl pyrimidine (HMP) pyrophosphate and 4-methyl-5-(β -hydroxyethyl)-thiazole (THZ) monophosphate. The THZ and HMP moieties are independently synthesized, and labeling studies have demonstrated probable metabolic precursors to both. We present herein the initial characterization of *thiI*, a gene required for THZ synthesis. We show that *thiI* is a 1,449-bp open reading frame located at minute 9.65 on the *S. typhimurium* chromosome and that it encodes a 483-amino-acid protein with a predicted molecular mass of 55 kDa. Unlike genes in the thiamine biosynthetic operon at minute 90, *thiI* is not transcriptionally regulated by TPP.

Thiamine pyrophosphate (TPP) is a required cofactor for a number of well-characterized enzymes (e.g., α -ketoglutarate dehydrogenase, pyruvate dehydrogenase, and transketolase). Although several recent studies of *Saccharomyces cerevisiae* (20–23), *Escherichia coli* (2, 28), and *Salmonella typhimurium* (9, 25, 29) have furthered our understanding of the synthesis of this vitamin, the genetic and biochemical characterization of the biosynthetic pathway is still in the early stages. In *S. typhimurium* and *E. coli*, thiamine monophosphate (TMP) is generated by the condensation of 4-amino-5-hydroxymethyl pyrimidine pyrophosphate (HMP-PP) and 4-methyl-5-(β -hydroxyethyl)-thiazole monophosphate (THZ-P) and then phosphorylated to TPP, the physiologically relevant form of the cofactor (Fig. 1). The HMP moiety of thiamine is derived from aminoimidazole ribotide, an intermediate in the purine biosynthetic pathway (10, 11) and a proposed intermediate in the alternative pyrimidine biosynthetic pathway (8, 26).

Labeling studies have demonstrated that the thiazole moiety is derived from tyrosine, cysteine, and 1-deoxy-D-xylulose in enteric bacteria. Tyrosine donates one carbon and one nitrogen atom, while cysteine is the source of the sulfur atom. The remaining atoms making up the thiazole moiety come from 1-deoxy-D-xylulose (6, 7, 12). Consistent with these data are at least two possible mechanisms for thiazole synthesis, one being pyridoxal phosphate dependent and the other being pyridoxal phosphate independent (3). Further work, including mutant analysis, is needed to correlate gene products with enzymatic activities and thus to elucidate the thiazole biosynthetic pathway.

A number of genes involved in the synthesis of thiamine have been identified, and their positions in the biosynthetic pathway are indicated in Fig. 1. In *E. coli* there are two kinase activities involved in the de novo synthesis of TMP. The genes encoding HMP kinase and HMP-P kinase are reported to map to minute 46 on the *E. coli* chromosome (*thiN* and *thiD*, respectively) (13, 18). It has recently been suggested that *thiN* be renamed *thiJ* (17). The *thiM* gene maps to a region similar to that of *thiN* and encodes thiazole kinase, a salvage enzyme which converts thiazole to thiazole monophosphate (16, 24).

Five additional genes, *thiCEFGH*, involved in thiamine biosynthesis have been mapped to minute 90 in both *E. coli* and *S. typhimurium* (28, 29). In *S. typhimurium*, these genes have been demonstrated to comprise an operon that is transcriptionally repressed in the presence of exogenous TPP (29). Biochemical functions for the gene products of two of these genes, *thiE* and *thiG*, have been determined. ThiE condenses THZ-P and HMP-PP in vitro to form TMP (2), and ThiG catalyzes an epimerization of deoxy-xylulose-5-phosphate, a putative intermediate in THZ synthesis (4). TMP, generated either de novo or through salvage of exogenous thiamine by the action of ThiK, is phosphorylated by TMP kinase (encoded by *thiL* at minute 10) to generate the active cofactor, TPP (14, 29).

We report here physical and genetic characterization of *thiI*, a new locus involved in THZ synthesis (29). Data presented here show that *thiI* is a 1,449-bp open reading frame (ORF) encoding a 55-kDa protein.

Isolation and characterization of *thiI* mutants. Seven independent insertions causing a Thi^- phenotype were isolated and mapped to a single locus at minute 10 on the *S. typhimurium* chromosome. The locus defined by these mutations was designated *thiI* (29). Strains carrying each of these insertions required exogenous thiamine or the thiazole moiety for growth. From this nutritional phenotype we concluded that *thiI* was required for thiazole synthesis in *S. typhimurium*. We also noted that under conditions where *thiI* mutants were grown with limiting thiamine, HMP was excreted into the growth medium (24). This result was consistent with previous data suggesting that HMP does not feedback inhibit its own synthesis (19) and supported a role for *thiI* in thiazole synthesis.

Sequence of *thiI*. A plasmid that complemented the THZ requirement of strain DM269 [*thiI887::Tn10d(Tc)*] was isolated from a pBR328 plasmid library (C. Miller, University of Illinois, Urbana-Champaign) and found to contain a 5.6-kb insert (pThiI). Two sequential subcloning steps described in Fig. 2 resulted in pThiI2.4, which contained a 2.4-kb insert that complemented all seven *thiI* insertions. The insert in pThiI2.4 was completely sequenced twice on one strand at the University of Wisconsin Biotechnology Center—Nucleic Acid and Protein Facility. Computer analysis of this sequence with BLASTN (1) aligned the first 367 bp of the insert with the *E. coli* sequence upstream of *xseB*. Since in *E. coli* this locus maps to minute 9.6, these data were consistent with P22 linkage data

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

FIG. 1. Proposed thiamine biosynthetic pathway in *S. typhimurium*. TPP is synthesized de novo through TMP and is composed of two moieties: THZ-P and HMP-PP. HMP-PP is derived from aminoimidazole ribotide (AIR), while THZ-P is derived from 1-deoxy-D-xylulose, L-tyrosine, and L-cysteine. Gene products, where known, are indicated at the appropriate step, and parentheses indicate uncertainty of the specific reaction catalyzed. APB, alternative pyrimidine biosynthetic pathway.

(50% linked to *apbA*) and definitively placed *thiI* at minute 9.65 on the *S. typhimurium* chromosome.

Further sequence analysis identified a 1,449-bp ORF within the insert that we subsequently demonstrated was *thiI* (see below). The nucleotide and predicted amino acid sequences of *thiI* are shown in Fig. 3. Critical to identifying this ORF as *thiI* was an internal *PstI* site (bp 1392) (Fig. 3), since a plasmid containing the insert truncated at this site was unable to complement any of the *thiI* mutants. Computer analysis predicted that the putative *thiI* ORF encoded a 483-amino-acid protein with a molecular mass of 55 kDa.

Mapping of *thiI* mutations by PCR. To confirm that the *thiI* insertions were in the 1,449-bp ORF designated *thiI*, four insertions were mapped by a PCR-based protocol described elsewhere (29). Primers specific for the appropriate insertion [Tn10d(Tc) or MudJ] (5) and for DNA 209 bp internal to the 1,449-bp ORF (5'-TCCCGGGGATTACCATA-3') (Fig. 3) were used to amplify the intervening DNA. When this protocol was performed with chromosomal DNA from DM269 (*thiI887::Tn10d*), DM410 (*thiI897::MudJ*), DM459 (*thiI909::MudJ*), and DM452 (*thiI902::MudJ*), amplified fragments of 1.1, 0.2, 0.8, and 0.9 kb, respectively, were obtained. These results clearly placed the four *thiI* insertions tested within the 1,449-bp ORF.

Overexpression of *ThiI*. To confirm that the 1,449-bp ORF encoded a protein of the predicted size, the *EcoRI-HindIII* fragment from pThiI2.4 was ligated into plasmids pT₇-5 and pT₇-6 (27) to generate pThiI-5 and pThiI-6, respectively. These plasmids were electroporated into *E. coli* BL21 *lde3* (containing T₇ RNA polymerase under the control of IPTG [isopropyl-β-D-thiogalactopyranoside]-inducible promoter) to produce strains DM2624 and DM2623, respectively. These strains were used for T₇-specific protein induction and [³⁵S] methionine labeling as described elsewhere (27). The proteins in the crude extract were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by exposure on a PhosphorImager screen.

Representative results of *ThiI* overexpression are shown in Fig. 4. The strain carrying pThiI-5 (with the *thiI* gene oriented for expression from the T₇ promoter) (Fig. 4, lane A) expressed a 56-kDa protein not represented in the control strain with pThiI-6 (lane B). Since a protein of the predicted size was produced, we concluded that *thiI* was a 1,449-bp ORF that encoded a 483-amino-acid protein with a molecular mass of 56 kDa required for THZ synthesis in *S. typhimurium*.

Transcriptional regulation. In contrast to transcription of the thiamine biosynthetic operon at minute 90 (29), transcription of *thiI* was not reduced by exogenous thiamine, TMP, or TPP when they were present in the medium at a concentration of 1 mM. Similarly, exogenous THZ had no effect on *thiI* transcription. β-Galactosidase activity was measured in either haploid (DM410, *thiI897::MudJ*, and DM66, *thiI882::MudJ*) or merodiploid (DM3200, *thiI897::MudJ*/pThiI2.4, and DM3199, *thiI882::MudJ*/pThiI2.4) strains containing a transcriptional fusion in *thiI*, as has been described previously (15). A basal level of ~70 U was found under all medium conditions tested.

It was interesting to note this difference in regulation between *thiI* and the thiamine biosynthetic genes at minute 90. Though we did not eliminate the possibility that *thiI* was tran-

FIG. 2. Cloning of *thiI*. Plasmid pThiI was isolated as described in the text. Digestion of pThiI with *SalI* (S) excised a 3.5-kb band that was ligated into pSUI9 to form pThiI3.5. Plasmid pThiI3.5 was cut with *EcoRI* (E) and self-ligated to form pThiI2.4, which complements all *thiI* mutants. pThiI2.4 contains a 2.4-kb insert with one 1,449-bp ORF that we have shown is *thiI*. Also on this plasmid is a part of the coding region of *xseB*, which maps to minute 9.6 in *E. coli*. The arrows represent directions of transcription. Boldface lines represent plasmid sequences. H, *HindIII*; P, *PstI*.

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