

Isolation and Characterization of New Thiamine-Deregulated Mutants of *Bacillus subtilis*

Ghislain Schyns,^{1*} Sébastien Potot,¹ Yi Geng,^{1†} Teresa M. Barbosa,² Adriano Henriques,² and John B. Perkins¹

Biotechnology R&D, DSM Nutritional Products, Ltd., Kaiseraugst, Switzerland,¹ and Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Oeiras Codex, Portugal²

Received 24 June 2005/Accepted 6 September 2005

In bacteria, thiamine pyrophosphate (TPP) is an essential cofactor that is synthesized *de novo*. Thiamine, however, is not an intermediate in the biosynthetic pathway but is salvaged from the environment and phosphorylated to TPP. We have isolated and characterized new mutants of *Bacillus subtilis* that deregulate thiamine biosynthesis and affect the export of thiamine products from the cell. Deletion of the *ydiA* gene, which shows significant similarity to the thiamine monophosphate kinase gene of *Escherichia coli* (*thiL*), did not generate the expected thiamine auxotroph but instead generated a thiamine bradytroph that grew to near-wild-type levels on minimal medium. From this Δ *thiL* deletion mutant, two additional ethyl methanesulfonate-induced mutants that derepressed the expression of a *thiC-lacZ* transcriptional reporter were isolated. One mutant, Tx1, contained a nonsense mutation within the *B. subtilis yloS* (*thiN*) gene that encodes a thiamine pyrophosphokinase, a result which confirmed that *B. subtilis* contains a single-step, yeast-like thiamine-to-TPP pathway in addition to the bacterial TPP *de novo* pathway. A second mutant, strain Tx26, was shown to contain two lesions. Genetic mapping and DNA sequencing indicated that the first mutation affected *yuaJ*, which encodes a thiamine permease. The second mutation was located within the *ykoD* cistron of the *ykoFEDC* operon, which putatively encodes the ATPase component of a unique thiamine-related ABC transporter. Genetic and microarray studies indicated that both the mutant *yuaJ* and *ykoD* genes were required for the derepression of thiamine-regulated genes. Moreover, the combination of the four mutations (the Δ *thiL*, *thiN*, *yuaJ*, and *ykoD* mutations) into a single strain significantly increased the production and excretion of thiamine products into the culture medium. These results are consistent with the proposed “riboswitch” mechanism of thiamine gene regulation (W. C. Winkler, A. Nahvi, and R. R. Breaker, *Nature* 419:952-956, 2002).

Vitamin B₁ (thiamine pyrophosphate), which can be synthesized by microorganisms, plants, and fungi but not by mammals, is a cofactor of a number of important enzymes in carbohydrate and amino acid metabolisms (30). Unlike other vitamin biosynthesis pathways in bacteria (e.g., for riboflavin and biotin), thiamine is not part of the *de novo* pathway but is actually part of the salvage pathway. In *Bacillus subtilis*, thiamine pyrophosphate biosynthesis occurs by a complex multi-step pathway (1). The pyrimidine moiety, 4-amino-2-methyl-5-hydroxymethylpyrimidine pyrophosphate (HMP-PP), is derived from an intermediate in the *de novo* purine biosynthetic pathway, 5-aminoimidazole ribotide, in a conversion catalyzed by the *thiC* gene product (1). HMP-P is then phosphorylated to HMP-PP by the product of the *thiD* (*yjbV*) gene prior to coupling with the thiazole unit (25). The thiazole moiety, 5-(2-hydroxyethyl)-4-methylthiazole phosphate (HET-P), is derived from 1-deoxy-D-xylulose 5-phosphate, glycine, and cysteine in a complex oxidative condensation reaction requiring the products of at least five different genes, *thiF*, *thiS*, *thiO*, *thiG*, and a *nifs*-like gene (26). The coupling of HMP-PP and HET-P is catalyzed by thiamine phosphate pyrophosphorylase encoded by *thiE*, resulting in thiamine monophosphate (TMP) (1). TMP

is then phosphorylated to form thiamine pyrophosphate (TPP) by the action of thiamine monophosphate kinase, encoded by *thiL*. *B. subtilis* contains another potential way to produce TPP, through a thiamine one-step salvage pathway formerly discovered in *Saccharomyces cerevisiae* (23). This pathway, initially thought to exist only in lower eukaryotes, is catalyzed by the product of the *thiN* (*yloS*) gene (19). Two other salvage kinases, one specific for hydroxyethylthiazole and the other with a broad range of substrate specificities, including vitamin B₆ compounds and HMP, have been identified in *B. subtilis* and are encoded by the genes *thiM* and *pdxK* (*ywdB*), respectively (25). However, an ortholog to the *E. coli* gene *thiK* (*ycfN*) encoding thiamine kinase, a salvage enzyme that converts thiamine to TMP, has not been identified in *B. subtilis* (19). The *B. subtilis* thiamine biosynthesis pathway showing all known and proposed genes and intermediates is shown in Fig. 1. (By recent convention, *B. subtilis thi* gene nomenclature has been changed to the *E. coli* nomenclature; some literature cited here may contain the original *B. subtilis thi* gene designations.)

Interestingly, a thiamine regulatory gene has not been identified so far in bacteria (9, 14, 38). Instead, it has been proposed that thiamine pyrophosphate directly regulates the expression of the thiamine biosynthesis genes by a novel mechanism called riboswitch, in which TPP interacts with the nascent mRNA message at a *cis*-acting site within the 5' leader region (called the *thi* box) to form a secondary structure that allows

* Corresponding author. Mailing address: Bldg. 203/25A, Wurmsweg 576, CH-4303 Kaiseraugst, Switzerland. Phone: 41 61 687-3034. Fax: 41 61 687-1847. E-mail: ghislain.schyns@dsm.com.

† Present address: Wyeth Pharmaceuticals, Pearl River, N.Y.

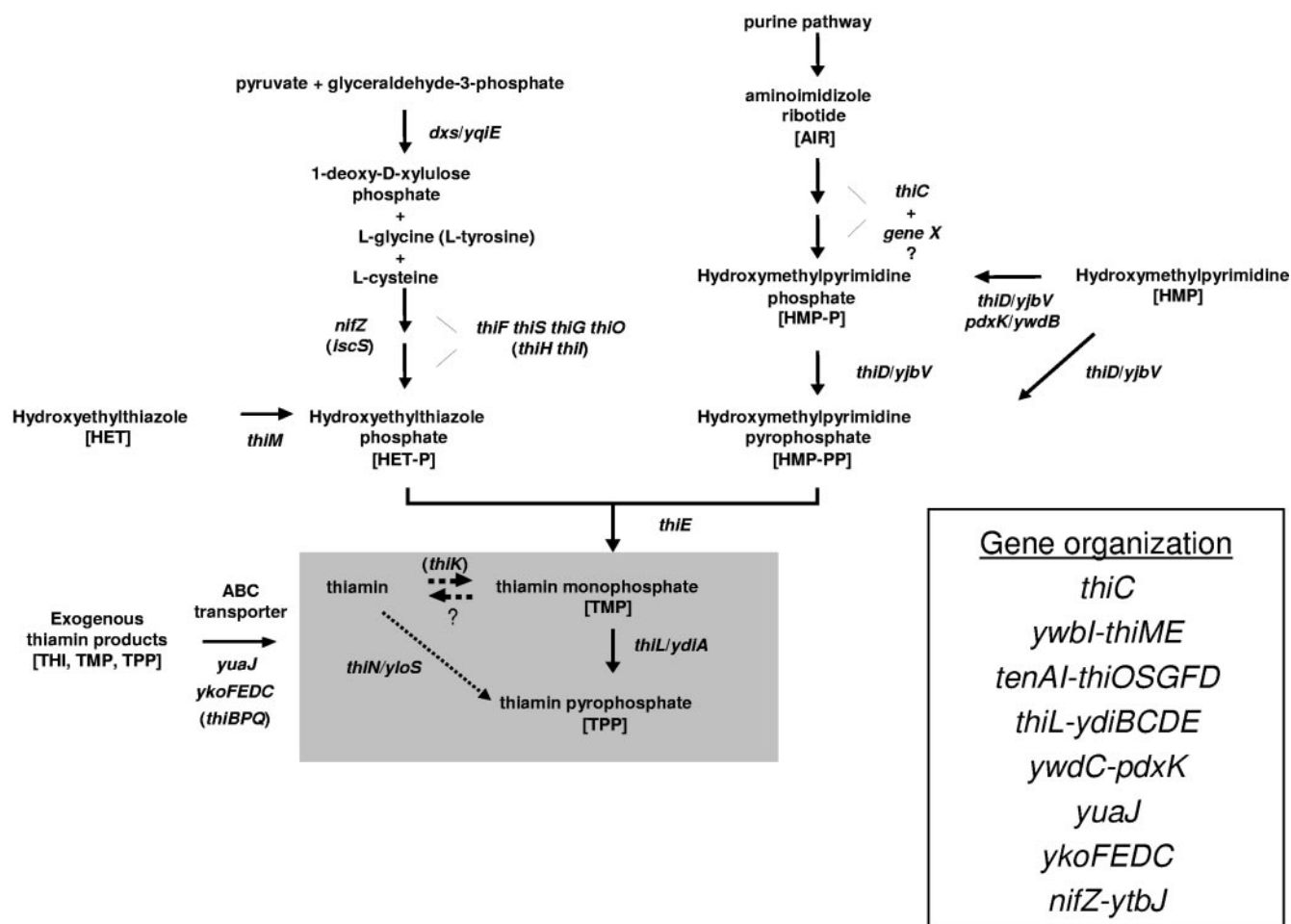


FIG. 1. Thiamine biosynthesis and salvage pathway of *Bacillus subtilis*. *B. subtilis* *thi* genes were previously identified by genetic, biochemical, and/or in silico evidence (1, 19, 25, 26, 29); in some cases, their original *y* gene designation is listed. *E. coli* intermediates or genes not present in *B. subtilis* but with similar functions are given in parentheses (1). Abbreviations are in brackets. The thiamine-TMP-TPP salvage pathway is shown in a shaded box. See the text for details. Question marks indicate possible unknown genes involved in the thiamine biosynthesis and salvage pathway (15, 19). The organization of *Bacillus* thiamine biosynthesis genes is shown on the right.

for the formation of a transcription terminator (17, 21, 22, 39). In addition to TPP, which was shown by transcription profiling to regulate the expression of the *thiC* gene and the *tenA-tenI-thiOSGFD* operon (16), thiazole (HET) was also described to repress the *ywbl-thiME* operon in *B. subtilis* (40, 41).

In silico screening of the *B. subtilis* genome with the *thi* box sequence has resulted in the identification of several new putative thiamine-regulated genes (29): the *yuaJ* gene and the *ykoF-ykoE-ykoD-ykoC* operon. *yuaJ* could encode an ATP-independent thiamine transporter containing multiple transmembrane segments. YkoE and YkoC are likely two transmembrane components, while YkoD is an ATPase component and YkoF acts as a novel HMP/thiamine-binding protein (6). However, no genetic experiments have confirmed the role of these genes in thiamine transport, and their products show no similarity to the known thiamine ABC transporter genes of *Salmonella enterica* serovar Typhimurium (*thiBPQ*) (36).

In this study, we used mutagenesis and transcriptomics to identify and characterize mutants of *B. subtilis* that deregulate thiamine gene expression. As predicted by the riboswitch

model of gene regulation, none of these mutations were located in genes predicted to encode a regulatory protein. Instead, these mutations mapped to several genes previously identified as encoding a thiamine pyrophosphokinase activity (*thiN*) or encoding thiamine-related transport proteins (*ykoD* and *yuaJ*). Moreover, we also show that these mutations must be combined to deregulate thiamine gene expression, resulting in bacterial strains that are altered in the production and export of thiamine. These results provide genetic support that intracellular TPP levels control thiamine biosynthesis gene expression.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Plasmids and strains used in this study are listed in Table 1. Antibiotic resistance genes that confer resistance to neomycin (*neo*), tetracycline (*tet*), and chloramphenicol (*cat*) were obtained from plasmids pBEST501, pDG1514, and pC194, respectively (*Bacillus* Genetic Stock Center, Ohio State University [BGSC]). *B. subtilis* was grown on minimal medium (MM; 1× Spizizen salts, 0.04% sodium glutamate, and 0.5% glucose) or veal infusion-yeast extract complete medium or grown on agar plates consisting of tryptone blood agar base (Difco, Maryland) or MM. *E. coli* was

TABLE 1. List of plasmids and strains

Strain or plasmid	Relevant characteristic or genotype	Reference
Plasmids		
pBR322	<i>E. coli</i> plasmid cloning vector	2
pMUTIN2	<i>B. subtilis</i> integration plasmid for gene inactivation	33
pDG1728	<i>B. subtilis</i> plasmid for ectopic integration of <i>lacZ</i> transcriptional fusions at <i>amyE</i>	10
pEpUCA1	<i>E. coli</i> - <i>B. subtilis</i> shuttle vector to generate in-frame mutations	31
pTH1	Internal 348-bp fragment of <i>thiL</i> (<i>ydiA</i>) cloned into pMUTIN2	This work
pTH12	712-bp <i>thiC</i> promoter fragment cloned into pDG1728	This work
pTH30	In-frame Δ <i>thiL</i> (<i>ydiA</i>) deletion (G ₇₉ -G ₂₀₂) cloned into pEpUCA1	This work
pTH31	Internal 353-bp <i>yuaJ</i> fragment cloned into pMUTIN2	This work
Strains		
<i>B. subtilis</i>		
PY79	SP β ^c (cured) prototroph	BGSC (1A747)
TH12	SP β ^c Δ <i>thiL::cat</i> ₄	This work
TH21	SP β ^c Ω <i>amyE::thiC-lacZ</i>	This work
TH22	SP β ^c Δ <i>thiL::cat</i> ₄ Ω <i>amyE::thiC-lacZ</i>	This work
TH48	Δ <i>thiL::cat</i> ₄ <i>yuaJ</i> (the first lesion in Tx26) <i>ykoD</i> (the second lesion in Tx26)	This work
TH76	Ω <i>yuaJ::pMUTIN2</i>	This work
TH83	Δ <i>thiL yuaJ ykoD</i>	This work
TH95	Δ <i>thiL thiN</i> (<i>tx1</i>) <i>yuaJ ykoD</i> Ω <i>yloA::Tn917</i>	This work
TH105	Δ <i>thiL::cat</i> ₄	This work
TH106	Δ <i>thiL::cat</i> ₄ Ω <i>thiL::pMUTIN2</i>	This work
TH112	Δ <i>thiL::cat</i> ₄ Ω <i>amyE::thiC-lacZ ykoD</i> Ω <i>yufR::Tn917</i>	This work
TH128	Δ <i>ispA::neo</i>	This work
TH137	Ω <i>yuaJ::pMUTIN2</i> Δ <i>ykoD::cat</i> ₄	This work
TH138	Δ <i>ykoD::cat</i> ₄	This work
1A603	(SP β c2) <i>trpC2</i> Ω <i>thiC84::Tn917</i>	34
1A631	(SP β c2) <i>trpC2</i> Ω <i>motA::Tn917</i>	34
1A633	(SP β c2) <i>trpC2</i> Ω <i>yloA::Tn917</i>	34
1A642	(SP β c2) <i>trpC2</i> Ω <i>yufR::Tn917</i>	34
Tx1	Δ <i>thiL::cat</i> ₄ Ω <i>amyE::thiC-lacZ thiN</i> (<i>tx1</i>)	This work
Tx26	Δ <i>thiL::cat</i> ₄ Ω <i>amyE::thiC-lacZ yuaJ ykoD</i>	This work
RL2066	Δ <i>spoVM::spec</i>	5
<i>E. coli</i>		
PT-R1	<i>thiO-35</i>	<i>E. coli</i> Genetic Stock Center; 13
K12	Wild type	<i>E. coli</i> Genetic Stock Center
<i>S. enterica</i> serovar Typhimurium		
DM456	Ω <i>thiD906::MudJ</i> (Km)	37
DM1856	Ω <i>thiL934::Tn10d</i> (Tc)	28

grown on MM or Luria-Bertani medium without glucose. The compositions of these media are described elsewhere (11). For testing thiamine production in liquid test tube cultures, a thiamine-free medium was used (7). Thiamine supplements (thiamine, TMP, and TPP) required for auxotrophy were added at 1 μ M. Resistance to the thiamine antimetabolite pyrithiamine was tested at 0.1 or 10 μ M.

Thiamine biological assays. Total thiamine compounds were assayed using indicator strains derived from *Salmonella enterica* serovar Typhimurium by known methods (7). Strain DM456 (Ω *thiD906::mudJ*) responds to thiamine, TMP, and TPP in MM, whereas strain DM1856 (Ω *thiL934::Tn10d*) responds to only TPP (28, 37). The responses of DM456 to known concentrations of thiamine, TMP, and TPP ranging from 0.0256 to 100 μ g/liter were similar. In addition, DM456 was found to be more sensitive to TPP than DM1856. To assay *B. subtilis* cultures, supernatants were filter sterilized before the preparation of dilutions. Intracellular thiamine levels were measured from dilutions of 1-ml filter-sterilized cellular extracts that were obtained by French press breaking of the cells and centrifugation at 10,000 \times g for 10 min. Indicator strains were grown overnight at 37°C in thiamine assay medium. Turbidity readings were made at 600 nm and compared to a range of standard solutions.

Thiamine thiochrome/HPLC assay. Individual thiamine compounds, thiamine, TMP, and TPP were measured using a modified thiochrome/high-performance liquid chromatography (HPLC) assay procedure described previously (3).

Briefly, 10 μ l of culture supernatant or intracellular extracts is added to 200 μ l of 4 M potassium acetate. The sample is then oxidized by the addition of 100 μ l fresh 3.8 mM potassium ferricyanide in 7 M NaOH. The mixture is vigorously mixed and then quenched by the addition of 100 μ l fresh 0.06% H₂O₂ in saturated KH₂PO₄. Samples are transferred to HPLC vials and injected onto a Supelcosil LC-18-T column (15 cm by 4.6 mm, 3 μ m) (Supelco reference no. 58970-U). Elution is made by a 10% to 35% methanol (H₂O, 50% to 25%) gradient in the presence of 40% 0.1 M K₂HPO₄ (pH 6.6) and 4 mM tetrabutyl ammonium hydrogen sulfate. Fluorescence is measured at 444 nm after excitation at 365 nm. The chronological order of elution from the column is thiamine, TMP, and TPP. This procedure was utilized to monitor both internal and external thiamine production during fermentation. In our HPLC/diode array detector assay used to directly measure thiamine and the intermediates HMP and HET in the fermentation broth, chromatography of samples was performed with a Phenomenex Luna C₁₈ column, using an Agilent 1100 HPLC system equipped with an autosampler plus thermostat and a diode array detector. The column dimensions are 150 by 4.6 mm, with a particle size of 5 μ m. The column temperature was kept constant at 20°C. The mobile phase is a mixture of 0.4 g pentane sulfonate in water, pH 2 (component A), and methanol (component B). Gradient elution, ranging from 2% (3 min) to 20% (20 min) component A, is applied. The flow rate is 1 ml/min. The detection method is based on UV absorption at 254 nm. The selectivity of the method was verified by injecting 10- μ l standard solu-

TABLE 2. List of PCR primers

Primer no.	Sequence
1.....	5'-GGGGGTACCGAAAATTGGATAAAGTGGG-3'
2.....	5'-GGGACGCGTAAGTACAGTCGGCATTATCTCATA-3'
3.....	5'-GGGGAATTCTACCAAGTTGTTCTGCCAAGGGCAT-3'
4.....	5'-GGGGTACCGCAAGTGATACCAGATAAAAACCTTAGG-3'
5.....	5'-GGGACGCGTCTTCAAAAATGGACTGAATCTGAAA-3'
6.....	5'-GGGGGATCCTTCAACGAGACAGACACCTTGTCCG-3'
7.....	5'-ATGCGGATCCCGTCCGACCGCC-3'
8.....	5'-CGATCCCGGGCCTCCCATCGCGG-3'
9.....	5'-ATGCCCGGGATTGCTAAGCTTCATCCTAAC-3'
10.....	5'-CGATGAATTCAGCCCTTCTGCAAAAACCTT-3'
11.....	5'-AGCTAAGCTTGGCAGCCGTTATTTAGACATTG-3'
12.....	5'-TGCAGGATCCATAAAAACCTGCGCTGACCACTGA-3'

tions of the relevant reference compounds, thiamine, HMP, and HET, each at 100 µg/ml. The target compounds were completely separated without special sample preparation.

Molecular and genetic techniques. Standard genetic and molecular biology techniques used in this study have been previously described by Maniatis et al. (18). DNA transformation, PBS1 generalized transduction, and other standard *B. subtilis* genetic techniques have been described previously (4).

Construction of *thiL* (*ydiA*) mutant. Comparison of the *E. coli* ThiL protein sequence to the SubtiList protein database detected significant similarity to only one protein, YdiA [$P(N) = 8.1e^{-15}$, where P is the probability of an alignment occurring with the score N or better]. The gene encoding this protein, *ydiA*, is 975 base pairs in length and is the first gene of a five-gene operon located at 55° on the *B. subtilis* chromosome. The *thiL* deletion mutant was generated by inserting a chloramphenicol acetyltransferase cassette (*cat₄*) lacking the endogenous *rho*-independent transcription termination site between nucleotides 267 and 272 of *thiL*. Removal of the terminator in *cat₄* results in nonpolar insertional mutations. To do this, PCR primer pair 1-2 (Table 2) was used to first generate the DNA cassette *cat₄* gene. This fragment was then individually ligated to two DNA fragments containing either the 3' or 5' ends of *thiL*, which were also generated by PCR using primer pairs 3-4 and 5-6 (Table 2). The PCR fragment containing the Δ *thiL::cat₄* cassette was then inserted directly into the chromosomal *thiL* gene of strain PY79 by DNA transformation, selecting for colonies resistant to 5 µg/ml chloramphenicol. Strain TH12 containing Δ *thiL::cat₄* was recovered.

Plasmid pEpUCΔ1 (31) was used to construct an in-frame, markerless deletion mutation in *B. subtilis*. This *E. coli* vector contains a selectable erythromycin resistance (*erm*) cassette and the pE194 temperature-sensitive origin of replication that does not function over 51°C. To do this, a DNA fragment containing an in-frame gene deletion was prepared using standard PCR methods and inserted between the BamHI and EcoRI sites of pEpUCΔ1. Competent cells of the recipient strain were transformed at 51°C with the in-frame mutation-containing plasmid pEpUCΔ1, selecting for erythromycin resistance at 5 µg/ml erythromycin. Em^r colonies that are also Cm^r were recovered and grown overnight at 28°C in the absence of antibiotic selection for 72 h. Bacteria were then plated onto tryptose blood agar plates, and the plates were incubated overnight at 37°C. Approximately 25% of the colonies were found to be sensitive to both erythromycin and chloramphenicol. PCR analysis of chromosomal DNA from several Em^s Cm^s colonies was used to confirm the presence of the in-frame mutation.

Isolation of EMS-induced thiamine-deregulated mutants. The strategy to isolate thiamine-deregulated mutants of *B. subtilis* was to mutagenize bacteria that contained a *thiC-lacZ* fusion and then screen for colonies that were Lac⁺ in the presence of TPP or thiamine. A 732-bp-long DNA fragment containing 417 bp of the 5' promoter region of *thiC* was prepared by PCR using standard methods and cloned unidirectionally in front of the promoterless *lacZ* gene of the pDG1728 vector (10), resulting in plasmid pTH12. This vector is designed to introduce ectopic transcriptional *lacZ* fusions into the nonessential *amyE* locus of *B. subtilis*. Plasmid pTH12 was linearized by restriction enzyme digestion and transformed into *B. subtilis* PY79, selecting for colonies that were resistant to 100 µg/ml spectinomycin (TH21). The same transcriptional fusion was also introduced into TH12 to generate TH22 (Δ *thiL::cat₄ amyE::thiC-lacZ*). Based on these results, *thiC-lacZ* reporter strain TH22 (Δ *thiL::cat₄*) was used to screen for deregulated mutants under repressing growth conditions. Two methods were used to isolate such mutants. In the first method, MM agar plates that contained 1 µM thiamine and 25 µg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were prepared. After a uniform dilution of logarithmic-growth-phase TH22 cells was applied, a paper disk containing 3 drops of ethyl methanesulfonate (EMS; $d = 1.17$ g/ml solution) was placed in the center of the plate. Lac⁺

colonies appeared over a period of 7 days of incubation at 37°C. In the second method, banks of EMS-mutagenized cells were prepared and screened. Accordingly, logarithmic-stage TH22 cells were treated with 9.4 mM EMS for 90 min, and aliquots were frozen in 10% glycerol at -90°C. Cells from the frozen stocks were diluted in veal infusion-yeast extract complete medium, incubated at room temperature for 30 min, and then plated onto MM containing 1 µM thiamine and 25 µg/ml X-Gal. Screening of Lac⁺ colonies led to mutants Tx1 and Tx26.

Combination of Δ *thiL* and the lesions in the mutant strains Tx1 and Tx26. As a first step, the mutations in Tx26 were transferred into TH12 (Δ *thiL::cat₄*) by DNA transformation, followed by selection for resistance to 0.1 µM pyrithiamine (P-0256; Sigma, St. Louis, MO). One pyrithiamine-resistant (Pyr^r) colony that was also Lac⁺ in the presence of TPP was recovered and designated TH48. Each strain was grown in MM supplemented with micronutrients and 2.5% Difco nutrient broth for 18 h at 37°C. The *cat*-interrupted *thiL* gene was next replaced by an in-frame deletion using pEpUCΔ1 (method described above). Using standard PCR methods, an in-frame deletion of *thiL* (removing amino acid residues Gly79 through Gly202) was first constructed using primer pairs 7-8 and 9-10 (Table 2) and inserted between the BamHI and EcoRI sites of pEpUCΔ1, creating pTH30. This plasmid was then used to replace the Δ *thiL::cat₄* mutation in TH48 with the in-frame *thiL* mutation (Δ *thiL*), resulting in strain TH83. The mutation in Tx1 (*thiN*) was next introduced into TH83 by PBS1 transduction by standard procedures using linkage to a silent Tn917 insertion, Ω *yloA::Tn917* (strain 1A633 of the *Bacillus* Genetic Stock Center, also called CU4153 [Ω *zdi-82::Tn917*]). The resulting strain was called TH95.

Mapping experiments and constructions of mapping strains. Genetic mapping studies using PBS1 generalized transduction were performed under standard conditions (4) using donor strains from the collection of phenotypically silent Tn917 mapping insertions (34) and strains listed in Table 1. In order to inactivate the *yuaJ* gene, a 324-bp internal fragment of *yuaJ* starting at position 353 was PCR amplified and inserted between the BamHI and HindIII sites of pMUTIN2 using primers 14 and 15 (Table 2), creating plasmid pTH31. To determine if the mutation in Tx1 is allelic to *thiN* (*yloS*), a 448-bp deletion mutation, starting at base 124 of the *thiN* gene (Δ *thiN::cat₄*), was constructed using the *cat₄* gene and cloned into pBR322 (2). Long-flanking-homology PCR was used to construct strain PY79 derivatives TH128 and TH138, which contain *cat₄*-mediated deletions of the *ispA* and *ykoD* genes, respectively (35).

Microarray profiling. TH22, Tx1, Tx26, and TH48 were grown in shake flask cultures containing 50 ml Spizizen MM with added thiamine pyrophosphate (0.34 µg/ml). Overnight cultures were diluted to 10 Klett units in fresh medium and grown to exponential growth phase (100 Klett units). Cells from half of the culture were collected by centrifugation, and the total RNA was immediately extracted as previously described (16). The remaining culture was grown to early stationary phase before RNA extraction. Early stationary phase was judged to be 30 min after glucose exhaustion; glucose content in the medium was measured by a glucose analyzer 2 (Beckman, Fullerton, CA) using standard procedures. The preparation of RNA, labeled cDNA targets, microarray hybridization and staining procedures, and data analysis used were described by Lee et al. (16).

RESULTS

Bacterial thiamine levels. As a first step in isolating *B. subtilis* thiamine deregulatory mutants, the intra- and extracellular levels of thiamine products from wild-type and engineered *B. subtilis* strains were determined as described in Materials and Methods. As a control, the thiamine-deregulated *E. coli* PT-R1 mutant was similarly tested; this mutant is reported to produce only intracellular thiamine (no excretion of thiamine products into the medium), mostly in the form of TPP (>90%). Bioassay results indicated that thiamine products were readily detected from extracts of sonicated cells but not from the culture medium (data not shown). The intracellular level of thiamine products in logarithmic- or stationary-phase wild-type *B. subtilis* was calculated to be approximately 100 to 200 µg/liter (Table 3). This thiamine level was lower than that for *E. coli* PT-R1, which reached approximately 1.6 mg/liter in stationary-phase cells. Determination of the individual thiamine forms using thio-

TABLE 3. Thiamine production and expression of *thiA-lacZ* transcriptional fusions in wild-type and thiamine-deregulated mutants of *B. subtilis*

Strain	Genotype	Accumulation ($\mu\text{g/liter}$)		Regulation (β -galactosidase sp act [Miller units]) ^c					
		Extracellular ^a	Intracellular ^b	Log			Stationary		
				None	HMP ^d	TPP ^d	None	HMP	TPP
TH21	<i>thiC</i> ⁺ Ω <i>amyE::thiC-lacZ</i>	<0.1	190	110	6	0.5	31	3	1.2
TH22	<i>thiC</i> ⁺ Ω <i>amyE::thiC-lacZ</i> Δ <i>thiL::cat</i> ₄	0.9	530	140	26	1.3	75	9	1.8
Tx1	<i>thiC</i> ⁺ Ω <i>amyE::thiC-lacZ</i> Δ <i>thiL::cat</i> ₄ <i>thiN</i>	5	500	NG	135	8	NG	177	5
Tx26	<i>thiC</i> ⁺ Ω <i>amyE::thiC-lacZ</i> Δ <i>thiL::cat</i> ₄ <i>yuaJ ykoD</i>	15	500	280	35	130	120	20	36

^a Thiamine concentration in MM (30 ml) after 24 hours of growth at 37°C.

^b Thiamine concentration of 1-ml supernatant of sonicated cells collected from a 30-ml MM culture after 24 hours of growth at 37°C.

^c Calculated according to Miller (20). NG, no growth.

^d Final concentrations of TPP and HMP were 1 μM each.

chrome/HPLC showed only trace amounts of TMP and TPP in the supernatant of PY79 cultures (<0.05 $\mu\text{g/ml}$). However, intracellular thiamine products were 80% TPP and 20% TMP, in good agreement with the bioassay results. Intra- or extracellular levels of free thiamine were not observed.

Construction and isolation of thiamine-deregulated mutants. The strategy to isolate and characterize thiamine-deregulated mutants was based on monitoring the activity of a *thiC-lacZ* transcriptional fusion in the presence of TPP or thiamine. As described in Materials and Methods, TH21 (Ω *amyE::thiC-lacZ*) is a derivative of PY79, in which a *thiC-lacZ* transcriptional fusion was integrated in single copy into the *amyE* gene. In control studies, TH21 displayed thiamine-regulated expression of the *lacZ* fusion. On MM agar plates containing the indicator X-Gal, colonies of TH21 were Lac⁺ (i.e., blue) but were Lac⁻ if the medium was supplemented with thiamine or TPP (1 μM). Moreover, when colonies were grown to early logarithmic phase (optical density at 600 nm of 0.8 to 0.9) in shake flask cultures containing 1 μM TPP, the β -galactosidase activity was repressed approximately 200-fold compared to cells grown in MM without TPP (Table 3). In stationary growth phase, the difference caused by TPP repression was less pronounced (30-fold repression). HMP (1 μM) also repressed expression of the fusion but to a lesser extent. Both thiazole and adenosine (1 μM each) did not show any thiamine-repressing activity (data not shown).

The *thiC-lacZ* transcriptional reporter fusion was introduced into a Δ *thiL::cat*₄ mutant (TH22) to screen for Lac⁺ (i.e., blue) colonies under thiamine- or TPP-repressing growth conditions. In *E. coli*, inactivation of the *thiL* gene produces a thiamine-deregulated strain which is a strict thiamine auxotroph (37). However, the *B. subtilis* Δ *thiL* mutant was not a thiamine or TPP auxotroph; instead, it appeared to be a thiamine bradytroph (data not shown) and was slightly deregulated for thiamine gene expression (Table 3). We chose this strain to generate thiamine-deregulated mutants in order to exclude *thiL* mutants from our screen. Out of 50,000 EMS-mutagenized colonies screened, 26 Lac⁺ mutants were recovered. Among these, two (Tx1 and Tx26) were selected for on the basis of their unique phenotypes. Tx1 appeared to be a thiamine bradytroph based on its slow-growth profile on MM without TPP. Conversely, Tx26 was a prototroph but conveyed a much stronger Lac⁺ phenotype than Tx1. In addition, Tx26 was found to

be resistant to pyrithiamine (Pyr^r), a toxic thiamine analog, at concentrations up to 10 μM .

Thiamine-deregulated mutations are allelic to genes that are involved in thiamine salvage and transport. PBS1 generalized transduction and DNA transformation were used to determine the genetic map positions of the lesions in Tx1 and Tx26. In most cases, the Tn917 mapping strains were used as donors; since these mapping strains carry the wild-type alleles, we reasoned that replacement of the mutation in Tx1 or Tx26 with the wild-type allele would result in a concomitant change in Lac phenotype (i.e., Lac⁺ to Lac⁻). In addition, the conversion of Pyr^r to Pyr^s could also be used to map the lesion(s) in the Tx26 mutant.

Initial mapping studies showed that the mutation in Tx1 was not linked to either the Δ *thiL::cat*₄ mutation or the Ω *amyE::thiC-lacZ* fusion. However, the Ω *spoVM::Tn917* mutation (strain RL2066) showed the highest transduction (>99%) and transformation linkages (72%) to the mutation in Tx1, indicating that this lesion was within or adjacent to *spoVM*. Inspection of this region located a possible candidate, *thiN* (*yloS*), a gene adjacent to *spoVM*, which has been previously shown to have thiamine pyrophosphokinase activity (thiamine-to-TPP activity) (19). To determine if the mutation in Tx1 is allelic to *thiN* (*yloS*), the DNA sequence of *thiN* in the Tx1 mutant was determined. The results revealed a single base mutation that caused a leucine-to-phenylalanine substitution at amino acid residue 116 (Leu₁₁₆ [CTT] > Phe₁₁₆ [TTT]).

Initial DNA transformation studies of Tx26 indicated that the transformation frequency of the pyrithiamine resistance marker to sensitive bacteria was nonlinear with respect to DNA concentration (data not shown). This result suggested that Pyr^r in Tx26 was caused by more than one mutation. Transduction experiments confirmed that the Tx26 mutant contained two mutations located at different regions of the chromosome. One mutation showed 70% linkage to Ω *yufR::Tn917*, and the other mutation showed 59% linkage to Ω *motA::Tn917*. Further transformation mapping studies showed strong linkage of the first lesion in Tx26 to *yuaJ*, a thiamine-regulated gene proposed to encode a thiamine permease (29). To determine if this lesion is allelic to *yuaJ*, a knockout mutation of *yuaJ* was first constructed using pMUTIN. As expected, the introduction of Ω *yuaJ::pMUTIN* into wild-type strains (e.g., PY79) or *thiC* mutants did not produce a phenotype. However, the introduction of Ω *yuaJ::pMUTIN* into

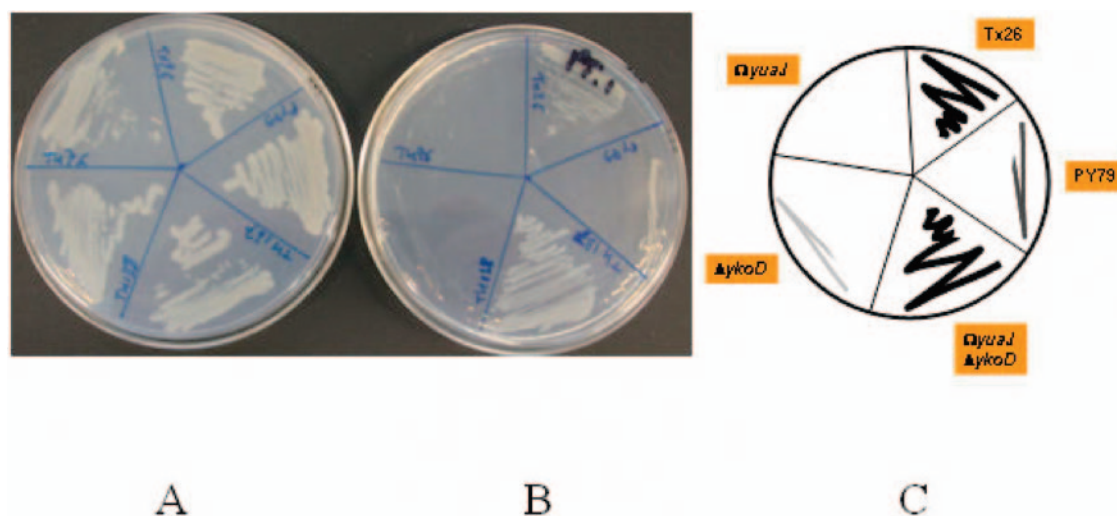


FIG. 2. Reconstitution of the Tx26 pyrithiamine resistance phenotype. The strains with the indicated mutations were streaked for single colonies on MM (A) or MM containing 0.1 μ M pyrithiamine and incubated for 3 days at 37°C (B). Panel C is a cartoon rendition of the results in panel B.

strain TH112 (with only the second Tx26 lesion and $\Delta thiL$) resulted in Em^r colonies that were resistant to 0.1 μ M pyrithiamine. Finally, DNA sequence analysis of *yuaJ* from Tx26 confirmed that the first lesion was an allele of *yuaJ*. YuaJ is a predicted membrane-bound thiamine permease. Sequencing detected a single base mutation that resulted in the change of a glutamine residue at amino acid position 35 to an ochre stop codon (Gln₃₅ [CAA] > Stop [TAA]). The introduction of the first Tx26 mutation is predicted to prematurely terminate translation, resulting in an inactive, 35-amino-acid truncated protein. In similar mapping studies, the second Tx26 allele showed strong linkage (65%) to the *ispA* locus (117°). This genetic map position corresponded to a cluster of genes, *ykoFEDC*, previously predicted to encode HMP transport proteins (29). DNA sequencing of PCR fragments containing this operon and the promoter region detected a single missense mutation in the *ykoD* gene that resulted in an Asp₁₈₀ (GAC)-to-Asn₁₈₀ (AAC) substitution.

Mutant *yloS* (*thiN*) has decreased pyrophosphokinase activity. Growth of Tx1 was found to be slower on MM lacking thiamine or TPP, taking more than 3 days to form colonies at 37°C. The addition of TPP or thiamine restored full growth within 1 day, suggesting that Tx1 was a strong thiamine bradytroph. In addition, Tx1 was strongly Lac⁺ on MM containing thiamine, HMP, or TMP but showed no Lac activity in the presence of TPP. These phenotypes strongly suggested that the lesion in the pyrophosphokinase gene decreases the conversion of thiamine to TPP whether thiamine is added externally or is formed by the dephosphorylation of TMP. Reconstruction of the Tx1 mutant confirmed this suspension. To do this, a double mutant (TH106) that consisted of a stable *thiN* deletion mutation ($\Delta thiN::cat_4$) and a *thiL* disruption mutation ($\Omega thiL::pMUTIN2$) was constructed. Growth on minimal or complex medium of this double mutant was similar to that displayed by the original Tx1 mutant; TH106 could grow on MM in the presence of TPP only, with the addition of thiamine or TMP failing to restore growth. The $\Delta thiN::cat_4$ mutation alone in

PY79 (TH105) produced no discernible phenotype in terms of growth on MM.

Simultaneous inactivation of YkoD and YuaJ is required for thiamine deregulation. Unlike Tx1, Tx26 containing mutant *ykoD* and *yuaJ* genes was a thiamine prototroph and grew like the parental strain on both minimal and complex media. Reconstruction experiments confirmed that the inactivation of both *yuaJ* and *ykoD* was required for resistance to pyrithiamine. To do this, a double mutant (TH137) that consisted of a stable *ykoD* deletion mutation ($\Delta ykoD::cat_4$) and a *yuaJ* disruption mutation ($\Omega yuaJ::pMUTIN2$) was constructed. This mutant was found to be resistant to pyrithiamine (Fig. 2). However, the *yuaJ* and *ykoD* single mutants were pyrithiamine sensitive. To further investigate thiamine deregulation in Tx26, β -galactosidase levels of the *thiC-lacZ* reporter were measured when Tx26 was grown under thiamine-repressing and -derepressing conditions. As shown in Table 3, β -galactosidase activity was induced more than 200-fold relative to that of the wild-type control when cells were grown to logarithmic stage in MM containing TPP. The level of deregulation was reduced to about 30-fold in stationary phase. In addition, β -galactosidase levels were only twofold higher in cells grown to logarithmic stage in MM without TPP than in cells grown with TPP (1 μ M); this difference in β -galactosidase levels was less pronounced in stationary growth phase. This variability in derepression of the *thiC-lacZ* fusion probably reflects fluctuation in TPP intracellular levels during growth. Interestingly, expression of the fusion was still partially repressed in the presence of HMP, which was likely caused by the conversion of HMP to TPP via the salvage pathway.

To determine if the expression of other thiamine biosynthesis genes was deregulated in Tx26, transcription profiling was carried out using a custom *Bacillus subtilis* oligonucleotide microarray (16). A reconstructed, backcrossed thiamine-deregulated mutant, TH48, was used in these first transcription profile experiments (see Materials and Methods) because the mutation(s) causing thiamine deregulation (i.e., pyrithiamine

TABLE 4. Change in transcript levels of genes in *B. subtilis* in response to TPP

Gene ^a	<i>thi</i> Box element ^c	Enzyme/function ^a	Ratio ^b	
			WT/WT ⁺ ^c	Dereg ⁺ /WT ⁺ ^d
<i>thiC</i>	+	Biosynthesis of hydroxymethylpyrimidine phosphate	58	105
<i>ywbI</i>	–	Similar to LysR family of transcriptional regulators	NC	NC
<i>thiM</i>	–	Hydroxyethylthiazole kinase	NC	NC
<i>thiE</i>	–	Thiamine phosphate synthase	NC	NC
<i>tenA</i>	+	Thiaminase ^f	68	62
<i>tenI</i>	+	Possible antagonist of TenA	25	27
<i>thiO</i> (<i>goxB</i>)	+	Glycine oxidase	67	56
<i>thiS</i> (<i>yjbS</i>)	+	Sulfur carrier protein	87	86
<i>thiG</i> (<i>yjbT</i>)	+	Thiazole synthase	82	61
<i>thiF</i> (<i>yjbU</i>)	+	ThiS adenyltransferase	87	96
<i>thiD</i> (<i>yjbV</i>)	+	Phosphomethylpyrimidine kinase	27	34
<i>pdxK</i> (<i>thiD</i>)	–	Pyridoxal/pyridoxal/pyridoxamine kinase	NC	NC
<i>thiL</i> (<i>ydiA</i>)	–	TMP kinase	NC	NC
<i>thiN</i> (<i>yloS</i>)	–	Thiamine pyrophosphorylase	NC	NC
<i>ytbJ</i>	–	Possible <i>thiI</i> orthologs (sulfur transferase)	NC	NC
<i>dxs</i> (<i>yqiE</i>)	–	1-Deoxy-D-xylulose synthase	NC	NC
<i>ykoC</i>	+	Possible transmembrane component of thiamine-related ABC transporter	10	15
<i>ykoD</i>	+	Possible ATPase component of thiamine-related ABC transporter	11	16
<i>ykoE</i>	+	Possible transmembrane component of thiamine-related ABC transporter	22	32
<i>ykoF</i>	+	Possible ligand-binding protein of thiamine-related ABC transporter	17	23
<i>yuaJ</i>	+	Possible thiamine permease	6.6	8.4
<i>yImB</i>	+	Similar to acetylornithine deacetylase	15	11

^a Gene names and functions were described by Perkins and Pero (27) and in SubtiList (<http://genolist.pasteur.fr/SubtiList>).

^b Transcript ratios were calculated by dividing the average difference values (after normalization) from hybridization experiments of wild-type cells grown to exponential phase in MM without TPP treatment by those with TPP treatment (WT[–]/WT⁺) or from hybridization experiments of deregulated mutant cells grown to exponential phase in MM with TPP treatment by those of wild-type cells grown under the same condition (Dereg⁺/WT⁺). For some genes (in bold), average difference values were obtained from duplicate probe sets per hybridization experiment: NC, no change in average difference values. Some data were taken from Table 1 in a report by Lee et al. (16).

^c *r* value of 0.95 (TPP-treated versus untreated data sets).

^d *r* value of 0.85 (TPP-treated TH48 versus TPP-treated PY79 data sets).

^e Under *thi* box element control according to report by Rodionov et al. (29).

^f See reference 32.

resistance) could be separated away from the *thiC-lacZ* fusion and any other unrelated EMS-induced mutation from the original Tx26 mutant (see below). Previous profiling results of wild-type *B. subtilis* PY79 demonstrated a 30- to 90-fold thiamine-specific repression of the two major thiamine biosynthesis operons, *thiC* and *tenAI-thiOSGFD*, but not of a third operon, *ywbI-thiME* (16). As shown in Table 4, high-level, deregulated expression of both *thiC* and *tenAI-thiOSGFD* was observed in TH48. Transcript levels of *thiC* increased 105-fold in TH48 compared to those in PY79 when both strains were grown in MM containing repressing levels of TPP. Transcription levels of *tenAI-thiOSGFD* increased 30- to 100-fold depending on the gene. The transcription of a third biosynthesis operon containing *thiM* and *thiE* and other known thiamine biosynthesis or salvage genes, *pdxK*, *dxs* (*yqiE*), *thiI* (*ytbJ*), and *thiN* (*yloS*), remained unchanged. A similar result was obtained by comparing transcription profiles of the original Tx26 mutant with those of the parental TH21 strain (data not shown). The Δ *thiL::cat₄* mutation in both TH48 and Tx26 had little effect on the transcription profile of thiamine biosynthesis genes other than *thiC* (a two- to threefold increase compared to PY79 when strains were grown to logarithmic stage in the presence of TPP) (data not shown). More interestingly, the transcript levels of genes recently shown to contain *thi* box regulatory sequences (29) also exhibited thiamine-specific derepression. For example, transcription of the *ykoFEDC* gene cluster was significantly increased in TH48 relative to PY79: *ykoF*, *ykoD*, and *ykoC* transcripts increased 15- to 23-fold, whereas *ykoE*

increased 32-fold. Other examples include the *yuaJ* gene and *yImB* encoding a protein similar to acetylornithine deacetylase. The average levels of *yuaJ* and *yImB* transcripts were 8- and 11-fold higher, respectively, in TH48 than in PY79. Similar patterns of regulated gene expression of *ykoFEDC*, *yuaJ*, and *yImB* were also observed in microarray analyses of wild-type cells grown in the presence or absence of thiamine. Finally, in HMP-grown TH48, the expression of only *thiC*, *thiOSGFD*, and *ykoFEDC* was repressed relative to cells grown in the absence of HMP (data not shown).

Strains with mutant *yloS*, *yuaJ*, and *ykoD* genes overproduce and excrete thiamine products. As shown in Table 3, there appeared to be a direct correlation between the degree of thiamine deregulation (i.e., expression level of the *thiC-lacZ* fusion in the presence of TPP) and the excretion of thiamine products. Strain TH22 with mutant *thiL* alone was only weakly thiamine deregulated and excreted the least amount of thiamine products relative to the TH21 wild-type control strain (ninefold higher). Tx1 with mutant *thiL* and *thiN* genes, which showed partial thiamine deregulation, produced a 50-fold-higher level of excreted thiamine products (5 μ g/liter). Lastly, Tx26, containing mutant *thiL*, *yuaJ*, and *ykoD* genes, which showed the highest level of thiamine deregulation, produced the highest level of excreted thiamine production (15 μ g/liter). This level of production was 100 to 150 times higher than that excreted by the control strain, and slightly over 50% of that excreted by Tx26 was in the form of TPP (data not shown). The level of intracellular thiamine production of all mutants was

TABLE 5. Thiamine production of TH95

Strain ^a	OD ₆₀₀ ^b	Concn produced (μg/liter)			
		Extracellular		Intracellular	
		Thiamine + TMP + TPP	TPP	Thiamine + TMP + TPP	TPP
Tx26 (Δ <i>thiL::cat₄ yuaJ ykoD amyE::thiC-lacZ</i>)	9.2	380	390	830 (25) ^c	370
TH95 (Δ <i>thiL thiN yuaJ ykoD YloA::Tn917</i>)	10.4	1270	120	1150 (40) ^c	270

^a Bacteria were grown in MM supplemented with 2.5% nutrient broth for 18 hours at 37°C. Culture media and cell-free extracts were measured for total thiamine products (thiamin + TMP + TPP) and TPP by a biological assay using *S. enterica* serovar Typhimurium indicators DM456 (*thiD906::MudJ*) and DMI856 (*thiL934::Tn10*), respectively.

^b OD₆₀₀, optical density at 600 nm.

^c Values in parentheses are the estimated extracellular concentrations of thiamine product if all are excreted into the culture medium in μg/liter; see Table 1.

approximately 500 μg/liter, or about threefold higher than that of the wild-type strain.

The four thiamine mutations, two in Tx26, *thiL*, and that in Tx1, were next combined into a single strain (as described in Materials and Methods) to determine if higher levels of thiamine overproduction and excretion could be obtained. Although Tx26 is a prototroph, the resulting strain (TH95) grew very poorly on MM unless TPP or nutrient broth (reported as containing significant levels of thiamine precursors HMP and thiazole [8]) was added to the medium. In this respect, TH95 was a stronger bradytroph than Tx1 (i.e., TH95 colonies took longer to appear on MM than Tx1 colonies). Despite this growth defect, TH95 produced threefold more excreted thiamine products in shake flask cultures than Tx26 (Table 5). Moreover, a shift in the excretion profile from predominantly TPP in Tx26 to a combination of thiamine and TMP in TH95 was observed. This shift in the thiamine product profile was confirmed by thiochrome/HPLC (data not shown).

DISCUSSION

It is now well accepted that “*thi* box” genes are regulated by a mechanism referred to as “riboswitch.” Using mainly *in vitro* methods, Winkler et al. (39) convincingly showed that TPP alone acts to regulate thiamine gene expression by directly interacting with the nascent RNA transcript at the *thi* box sequence to block either transcription or translation. The work presented here describes mutagenesis studies that identified and characterized new thiamine-deregulated mutations of *Bacillus subtilis* that are located within the coding regions of three genes: *thiN*, *yuaJ*, and *ykoD*. The encoded proteins have been lately suggested by *in silico* analysis to be involved in thiamine salvage and transport. We demonstrate here that indeed an important phenotype of these deregulatory mutants is the ability to excrete significant quantities of thiamine products and, therefore, that an important role of these proteins seems to be the control of TPP levels. It is important to note that the identification of deregulatory mutations within thiamine transport genes is strikingly similar to the recent results of Johansen et al. (12) and Nygaard and Saxild (24), which showed that mutations that increase the activity of the *pbuE*-encoded purine-specific pump deregulate expression of the G-box regulon by increasing the efflux of hypoxanthine, a metabolite that regulates purine gene expression by a riboswitch mechanism.

Melnick et al. (19) first demonstrated that *B. subtilis* contained a thiamine pyrophosphorylation activity normally found

in yeast. This activity was encoded by *yloS*, subsequently renamed *thiN*. We present here experimental evidence confirming the function of this enzyme in thiamine biosynthesis. Indirect genetic evidence for this gene came from mutagenesis studies of the *B. subtilis thiL* gene, which resulted in mutants that were thiamine bradytrophs rather than strict auxotrophs, as shown for *thiL* mutants of *S. enterica* serovar Typhimurium and *E. coli*. This shows that *B. subtilis* has two pathways to produce TPP. Direct evidence was obtained by showing that the mutation in Tx1 mapped to *thiN* and that reconstitution experiments indicated that inactivation of both *thiN* and *thiL* together resulted in a synthetic TPP auxotrophy in *B. subtilis*. In addition, the differential Lac activity displayed by Tx1 in the presence of thiamine (Lac⁺) and TPP (Lac⁻) is consistent with the presence of a direct thiamine-to-TPP route (Table 3). By blocking the ThiL reaction (TMP to TPP) and reducing the ThiN (YloS) reaction (THI to TPP) by the Tx1 mutation, the expected intracellular TPP level would be too low to repress *thi* gene expression. What is not clear, however, is whether these two pathways are linked (Fig. 1). Preliminary genetic studies suggest that *B. subtilis* contains both thiamine kinase and TMP phosphatase activities (data not shown). However, because the *B. subtilis* genome does not contain genes that display significant similarity to known thiamine kinase (e.g., *E. coli thiK* [*ycfN*]) or TMP phosphatase (yeast PHO3) genes, it will be interesting to determine if these putative enzymes are specific to thiamine biosynthesis or represent activities from house-keeping or other biosynthesis pathways.

The mutations in the Tx26 mutant were found to be allelic to two putative thiamine transport genes, *yuaJ* (the first lesion) and *ykoD* (the second lesion). The *yuaJ* gene is predicted to encode a thiamine permease (passive transport) that contains six predicted *trans*-membrane domains (29). The *ykoD* gene is predicted to encode an ATPase activity, which together with the *ykoC* and *ykoE* gene products is predicted to form an ABC transporter (29). The Tx26 mutations were found to generate three phenotypes: deregulation of thiamine gene expression, an increase in pyrithiamine resistance, and an increase in extracellular levels of thiamine products. The fact that inactivation of both *yuaJ* and *ykoD* was required to cause all three phenotypes suggested that these transporters control the influx of TPP and other thiamine products into the cell (mutations that inactivate efflux transporters would not be consistent with our observation of higher extracellular levels of thiamine product with Tx26). Based on these results, we recommend that *yuaJ*

be renamed *thiT* and that the *ykoFEDC* operon be renamed *thiUVWX*. Although in silico analysis does not indicate a function for *ykoF*, we have tentatively designated it a *thi* gene because it is cotranscribed with the *thiVWX* genes and because the crystal structure of its encoded protein indicates that it binds thiamine molecules (6).

Both *lacZ* reporter fusion (Table 3) and microarray experiments indicated that the expression of all known *thi* box-containing genes (e.g., *thiC*, *thiOSGFD*, *ykoFEDC*, and *yuaJ*) was highly derepressed in the Tx26 mutant. Since a key phenotype of this mutant is the efflux of thiamine products from the cell, gene deregulation is most likely caused by changes in TPP intracellular levels. Interestingly, the addition of exogenous HMP appeared to partially regulate thiamine gene expression (Table 3) in Tx26. We do not believe HMP is functioning as a repressor (or corepressor) independent of TPP because *lacZ* reporter fusion experiments showed that HMP did not repress *thiC* expression in the Tx1 mutant. It seems more likely that HMP enters the cell by a different transport route and is converted to the TPP effector via the salvage pathway (HMP to HMP phosphate to TMP to TPP), which then interacts with *thi* box sequences of the nascent mRNA to repress *thi* gene expression via the riboswitch mechanism.

Finally, an important application of our study was that these thiamine-deregulated mutants could be further manipulated to generate new strains that excrete more thiamine products. The combination of four mutations in *thiL*, *thiN*, *thiT* (*yuaJ*), and *thiW* (*ykoD*) has led to a strain that under 10-liter-scale fermentation conditions excreted thiamine products in the mg/liter range (data not shown). Genetic engineering of the thiamine biosynthesis and precursor genes should lead to strains with a further increase in thiamine production.

ACKNOWLEDGMENTS

We gratefully acknowledge Werner Bretzel for analytical support, Markus Goese for fermentation support, and Jian-ming Lee for microarray analysis of thiamine-deregulated mutants. We thank Hans-Peter Hohmann for helpful discussion and critical reading of the manuscript. We also thank Tadhg Begley and Diana Downs for providing data before publication and for their expertise and support during the course of this work.

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