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# Thiamine Triphosphate, a New Signal Required for Optimal Growth of *Escherichia coli* during Amino Acid Starvation*

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**Bernard Lakaye‡, Barbara Wirtzfeld‡§, Pierre Wins¶, Thierry Grisar, and Lucien Bettendorff‖**

*From the Center for Cellular and Molecular Neurobiology, University of Liège, 17, place Delcour, 4020 Liège, Belgium*

**Thiamine triphosphate (ThTP) is present in low amounts in most organisms from bacteria to humans, but its biological role remains unknown. *Escherichia coli* grown aerobically in LB medium contain no detectable amounts of ThTP, but when they are transferred to M9 minimal medium with a substrate such as glucose or pyruvate, there is a rapid but transient accumulation of relatively high amounts of ThTP (about 20% of total thiamine). If a mixture of amino acids is present in addition to glucose, ThTP accumulation is impaired, suggesting that the latter may occur in response to amino acid starvation. To test the importance of ThTP for bacterial growth, we used an *E. coli* strain overexpressing a specific human recombinant thiamine triphosphatase as a glutathione *S*-transferase (GST) fusion protein (GST-ThTPase). Those bacteria were unable to accumulate measurable amounts of ThTP. On minimal medium supplemented with glucose, pyruvate, or acetate, they exhibited an intermediate plateau in cell growth compared with control bacteria expressing GST alone or a GST fusion protein unrelated to thiamine metabolism. These results suggest that the early accumulation of ThTP initiates a reaction cascade involved in the adaptation of bacteria to stringent conditions such as amino acid starvation. This is the first demonstration of a physiological role of this ubiquitous compound in any organism.**

Thiamine triphosphate (ThTP),[1] the triphosphorylated derivative of vitamin B1, is a minor component of most cells (generally 0.1–1% of total thiamine). Yet it has been found in most organisms from prokaryotes to mammals (1). In contrast to the well known coenzyme role of thiamine diphosphate (ThDP), the biological role of ThTP has remained elusive so far. Recent findings, however, suggest that ThTP phosphorylates certain proteins, and this might be part of a new cellular signaling pathway. The most significant finding (2) was that, in *Torpedo marmorata* electric organ, ThTP specifically phosphorylates rapsyn, a protein essential for the clustering of acetylcholine receptors at the neuromuscular junction (3). Interestingly, ThTP phosphorylated histidyl residues on rapsyn. Histidyl phosphorylation is a major regulatory event in prokaryotes (4) but is less well established in animals (5). In the latter case the rule is phosphorylation on serine, threonine, and tyrosine with ATP as the phosphate donor. In primitive microorganisms, however, there are apparently more variations on this theme, and proteins may be phosphorylated by donors others than ATP; for instance, phosphoenolpyruvate is the phosphate donor for the phosphoenolpyruvate-sugar phosphate transferase of *Escherichia coli* (6). It has also been reported (7) that CheY, the response regulator that controls motility in chemotaxis, can be autophosphorylated by low molecular weight metabolites such as acetyl phosphate and carbamoyl phosphate.

It is, thus, tempting to speculate that protein phosphorylation by ThTP is also an ancient process and that cell signaling mechanisms involving ThTP may be more important in microorganisms than, for instance, in mammals. This prompted us to investigate ThTP biosynthesis in bacteria. As we have recently reported, *E. coli* grown aerobically in a LB medium under optimal conditions contain no detectable amounts of ThTP (1). However, measurable amounts appeared when the bacteria were switched from aerobic to anaerobic conditions. It was, thus, interesting to investigate in more detail the relationship between ThTP biosynthesis and adaptation of *E. coli* to changing metabolic conditions and growth. Here we show that when bacteria are transferred from LB medium to a minimal medium containing glucose or pyruvate but no amino acids, ThTP levels rapidly and transiently increase (up to 20% of total thiamine). Furthermore, bacteria rendered ThTP-deficient by overexpression of a specific mammalian ThTPase displayed an intermediate plateau in growth rate under the conditions that normally lead to ThTP synthesis. The results suggest that ThTP synthesis is a signal facilitating the adaptation of bacteria to stringent conditions such as amino acid starvation, possibly through protein phosphorylation.

## EXPERIMENTAL PROCEDURES

*Growth and Processing of Bacteria*—The *E. coli* strains used were either MG1655 (wild-type K-12 strain), CF5802 (Δ*ppk-ppx*::*km*) (8), or BL21 (Amersham Biosciences). BL21 were transformed with pGEX-5X-1 encoding either glutathione *S*-transferase (GST), the GST-ThTPase fusion protein (9), or the GST-EFHC1 fusion protein (see next paragraph). The bacteria were grown overnight (37 °C, 250 rpm) in 50–100 ml of Luria-Bertani (LB) medium (Tryptone, 10 g/liter; yeast extract, 5 g/liter; NaCl, 10 g/liter, at pH 7.0). Then the bacteria were centrifuged (5 min; 10,000 × *g*) and suspended in the initial volume of fresh LB medium or M9 minimal medium ($Na_2HPO_4$, 6 g/liter; $KH_2PO_4$, 3 g/liter; NaCl, 0.5 g/liter; $NH_4Cl$, 1 g/liter; $CaCl_2$, 3 mg/liter; $MgSO_4$, 1 mM, pH 7.0) containing various metabolic substrates. If not otherwise stated, the bacteria were incubated for 30 min at 37 °C with shaking (250 rpm). For transformed bacteria, ampicillin (200 μg/ml) was present in the culture medium at all stages. After incubation, the bacteria were sedimented as above, the pellets were suspended in 12% trichloroacetic

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‡ These authors equally contributed.

§ A graduate student of the Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture.

¶ Research Associate at the Fonds National de la Recherche Scientifique (FNRS).

‖ Senior Research Associate at the FNRS. To whom correspondence should be addressed. Tel.: 32-4-366-59-67; Fax: 32-4-366-59-53; E-mail: L.Bettendorff@ulg.ac.be.

[1] The abbreviations used are: ThTP, thiamine triphosphate; ThDP, thiamine diphosphate; ThTPase, thiamine triphosphatase; GST, glutathione *S*-transferase; polyP, inorganic polyphosphate; IPTG, isopropyl-1-thio-β-D-galactopyranoside.

TABLE I
*Effect of carbon source on ThTP and ATP contents of E. coli transferred to minimal M9*

After overnight culture in LB medium, the bacteria were resuspended in the initial volume of M9 minimum medium and incubated for 30 min at 37 °C in the presence of various substrates at a final concentration of 10 mM. The number of experiments is indicated in parentheses. For ATP, statistically significant differences were assessed using the Kruskal-Wallis nonparametric test ($p < 0.0001$) followed by Dunn's multiple comparisons test for comparison with the control. ND, not detectable.

| Carbon source | ThTP | ATP |
|---|---|---|
| | *pmol/mg of protein* | *nmol/mg of protein* |
| No substrate (16) | ND | 1.3 ± 0.5 |
| Glucose (12) | 60 ± 21 | 3.2 ± 1.6[a] |
| Fructose (3) | 50 ± 6 | 3.4 ± 0.1 |
| Gluconate (6) | 50 ± 7 | 2.7 ± 0.8[b] |
| Pyruvate (28) | 90 ± 39 | 2.5 ± 0.7[a] |
| Acetate (3) | 50 ± 9 | 0.4 ± 0.1 |
| Glycerol (3) | 13 ± 1 | 0.55 ± 0.14 |
| Citrate (3) | ND | 1.7 ± 0.2 |
| Oxoglutarate (3) | 17 ± 1 | 1.2 ± 0.1 |
| Glutamate (5) | ND | 0.79 ± 0.01 |
| Succinate (7) | 19 ± 12 | 1.4 ± 0.7 |
| Fumarate (3) | ND | 2.9 ± 0.1 |
| Malate (6) | ND | 3.8 ± 1.1[b] |
| Oxaloacetate (3) | ND | 4.1 ± 0.2[b] |

[a] $p < 0.01$.
[b] $p < 0.05$.

acid, the precipitated proteins were spun down (15 min, 10,000 × *g*), and the pellet was dissolved in 0.8 N NaOH for protein determination by the method of Peterson (10). The supernatant was treated with diethyl ether and analyzed by high pressure liquid chromatography for thiamine compounds (11) and ATP (12). The authenticity of ThTP synthesized was checked by purification on an AG-50W-X8 ($H^+$ form, Bio-Rad) cation exchange resin and subsequent hydrolysis by purified bovine ThTPase as previously described (1).

*Construction of GST-EFHC-1*—EFHC1 (GenBank™ accession number BC020210) codes for a eukaryotic protein with a single EF-hand domain. Its open reading frame was amplified from human brain cDNA using primers EFHC1for (5′-GAATTCATGGTGTCCAATCCCGTGCATG-3′) and EFHC1rev (5′-CTCGAGTCAGTTTGAGAAAGCACGAAC-3′) and *Pfx* DNA polymerase (Invitrogen). The PCR fragment was first cloned into pCR4 using the zero blunt TOPO PCR cloning kit (Invitrogen). The fragment was isolated by XhoI and EcoRI digestion and inserted into the GST expression plasmid pGEX-5X-1 (Amersham Biosciences). Expression of the full-length protein was checked after IPTG induction by SDS-PAGE followed by Coomassie blue staining.

## RESULTS

*ThTP Synthesis in E. coli Depends on the Metabolic Status*—We have previously shown that when *E. coli* BL21 bacteria were grown under optimal conditions in LB medium (presence of oxygen and vigorous stirring), ThTP levels were virtually undetectable (1). However, when the bacteria were grown in the absence of oxygen, ThTP could be detected. On the other hand, in the only other report on the presence of ThTP in *E. coli*, the bacteria were grown in LB medium supplemented with glucose (13). This suggested that a relationship exists between glycolysis and ThTP synthesis.

LB broth is an undefined complex medium mainly composed of amino acids (Tryptone, a pancreatic digest of casein), yeast extract, and NaCl, and we therefore switched to the minimal medium M9 supplemented with various carbon sources (10 mM). ThTP and ATP were determined after 30 min in the defined minimal medium M9 under aeration (Table I). No significant amount of ThTP could be detected in the absence of a carbon source, although the bacteria still contained appreciable amounts of ATP. Glucose, fructose, gluconate, pyruvate, and acetate induced an important increase in intracellular ThTP levels. ThTP was identified after purification and hydrolysis by a specific ThTPase as previously described (1). A smaller amount of ThTP was observed in the presence of glycerol, oxoglutarate, and succinate. Other Krebs cycle intermediates such as citrate, fumarate, oxaloacetate, and malate were completely ineffective, although especially the latter two significantly raised bacterial ATP content. These results suggest that ThTP accumulation is not directly related to the cellular ATP concentration but that the important factor is the nature of the carbon source. This hypothesis is supported by the observation that acetate is a good inducer of ThTP accumulation, although the ATP content remains low.

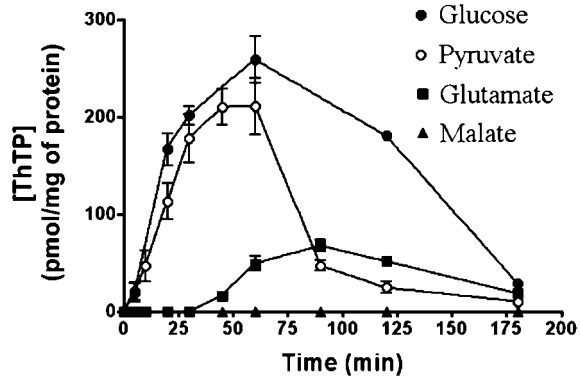


FIG. 1. **Kinetics of ThTP accumulation by *E. coli* in the presence of various carbon sources.** The bacteria from an overnight culture (20–50 ml) were centrifuged and suspended in the initial volume M9 minimum, and aliquots of 2 ml were incubated (37 °C, 250 rpm) in the presence of 10 mM glucose, pyruvate, glutamate, or malate. After various periods of time the bacteria were sedimented (10,000 × *g*, 5 min), and the pellet was treated with 1 ml of 12% trichloroacetic acid for the determination of ThTP. The results are expressed as mean ± S.D. for 3–6 experiments.

The previous results suggest that ThTP accumulation is dependent on the metabolic status of the bacteria. Glucose, fructose, gluconate, and pyruvate directly feed into the glycolytic pathway, yielding acetyl-CoA. In our experiments, glucose and pyruvate were as effective in raising ThTP concentrations in the presence and the absence of oxygen (not shown). In *E. coli*, pyruvate will yield acetyl-CoA even in the absence of oxygen, due to the existence of a pyruvate-formate lyase (14). Acetyl-CoA can then be processed to acetyl phosphate and acetate with synthesis of ATP. Thus, oxidative metabolism does not appear to be required for ThTP accumulation. This is in agreement with the observation that in LB medium moderate ThTP levels were observed under anaerobic conditions (1).Bacteria fed on acetate accumulate ThTP, and acetate can only be metabolized to acetyl-CoA that is further metabolized via the glyoxylate shunt and the tricarboxylic cycle (15).

Krebs cycle intermediates will not rapidly yield high amounts of acetyl-CoA, and they do not induce the accumulation of a significant amount of ThTP, with two notable exceptions, oxoglutarate and succinate. It may be significant that both compounds are able in one step to yield another thioester, succinyl-CoA, the former via oxoglutarate dehydrogenase and the latter via succinyl-CoA synthetase. Thus, all our results suggest a link between thioesters and ThTP levels.

*ThTP Accumulation Is Initiated in Minimal Medium within Minutes after the Addition of a Suitable Substrate*—As shown in Fig. 1, ThTP was detected already 5 min after the addition of glucose or pyruvate (10 mM) to the minimal M9 medium. The maximum (about 200–250 pmol·mg$^{-1}$) was reached after 40–60 min, and ThTP levels started to decrease after 1 h. In the presence of the same concentration of glutamate, the appearance of ThTP was strongly delayed, and the maximum amount obtained remained much lower than in the presence of glucose or pyruvate. In the presence of malate no significant

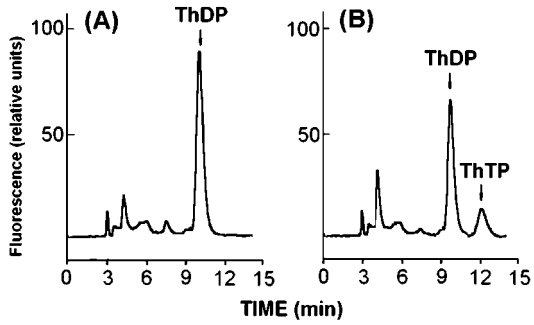


FIG. 2. **Chromatograms showing the appearance of ThTP.** Bacteria from an overnight culture were shifted to M9 minimum medium, and aliquots (2 ml) were incubated for 30 min either in the absence (*A*) or presence (*B*) of 10 mM pyruvate as described in legend to Fig. 1. ThDP and ThTP peaks are indicated.

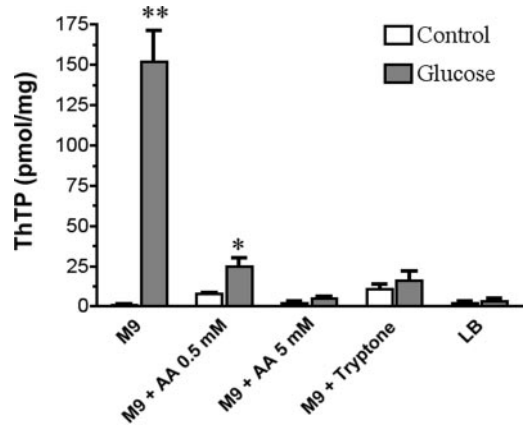


FIG. 3. **Effect of medium composition on the accumulation of ThTP in the absence or the presence of glucose (10 mM).** The bacteria were prepared as described in legend to Fig. 1 and incubated for 30 min either in LB medium or in M9 medium supplemented with a mixture of amino acids (*AA*) or with Tryptone (10 g/liter as in LB medium). The amino acid mixture contained all 20 amino acids, each at either 0.5 or 5 mM (except for the poorly soluble Tyr, which was at 0.5 mM). Shown are Kruskal-Wallis nonparametric test ($p < 0.0001$) followed by Dunn's multiple comparisons test for comparison between control and glucose (*, $p < 0.05$; **, $p < 0.01$).

amount of ThTP was observed. Interestingly, when pyruvate was added to the LB medium, a maximum ThTP content of about 30–35 pmol·mg$^{-1}$ was reached after 90 min (not shown). This value is 5–10 times lower than the one obtained in M9 medium. No significant accumulation was observed in the presence of glucose in LB medium. Under these conditions the bacteria remained in a stationary phase, and no significant bacterial growth was observed within the 3-h incubation period. This is probably due to the very high bacterial density under these conditions ($A_{600} > 2$).

After 60 min in M9 medium containing glucose, the ThTP content reached about 250 pmol·mg$^{-1}$, *i.e.* 20% percent of total thiamine (Fig. 2). The intracellular concentration was estimated to be about 20 $\mu$M at the maximum of the peak. During the same period ThDP content decreased from 1208 ± 36 to 978 ± 73 pmol/mg of protein ($n = 8$; Student's $t$ test: $p = 0.03$), suggesting that ThTP is synthesized from an intracellular ThDP pool.

*ThTP Levels Are Linked to Amino Acid Starvation*—Our results show that ThTP accumulation is dependent on two parameters that act synergistically, (i) the presence of the suitable carbon source and (ii) transfer to a minimal medium, lacking amino acids but also all vitamins. To test whether the absence of amino acids is responsible for the accumulation of ThTP induced by glucose, the bacteria were grown in M9 medium in which various concentrations of amino acids or Tryptone were added (Fig. 3). It can be seen that the presence of the 20 amino acids at 0.5 mM each was enough to nearly completely antagonize the effect of glucose. To check if one or several particular amino acids were responsible for the effect observed in Fig. 3, all 20 amino acids occurring in proteins were tested separately (Fig. 4). At 10 mM, many amino acids caused a small (10–20%) reduction of glucose-induced ThTP accumulation compared with the control (no amino acid present). Some were more effective; arginine, glutamate, tryptophan, and cysteine caused a 50–70% decrease in ThTP levels. These results suggest that in *E. coli* ThTP accumulation does not occur in response to the absence of one particular amino acid but, rather, in response to general amino acid starvation.

It is well known that inorganic polyphosphate (polyP) plays a role in the adaptation of the bacteria transferred from a rich to a minimal medium as it stimulates endogenous protein degradation to increase the availability of amino acids (16). PolyP promotes the degradation of ribosomal proteins by the Lon protease (17). However, the bacterial strain BL21 used here lacks protease Lon (that is the reason why this strain is used to increase the efficiency of protein expression). If ThTP is involved in the response to amino acid starvation in addition to polyP, it seemed important to check whether wild-type K-12 bacteria (MG1655) and also CF5802 bacteria, lacking polyP kinase and exopolyphosphatase, still produce ThTP when fed on pyruvate and glucose in minimal medium. The wild-type strain produced the same amount of ThTP as the BL21 strain, but ThTP accumulation was twice as high in the CF5802 strain compared with the previous strains (not shown). This could be because of the absence of exopolyphosphatase, which might be able to hydrolyze ThTP. Taken together, these data suggest that neither polyP nor polyP kinase is implicated in ThTP accumulation.

*In the Absence of Amino Acids, Bacteria Lacking ThTP Show an Intermediate Plateau in Growth Rate*—The data presented above show that a transient accumulation of ThTP occurs in response to the transfer of bacteria from LB to M9 medium (containing an appropriate carbon source but no amino acids), and it is, thus, possible that ThTP may play a role in this adaptation. To test this hypothesis we compared the growth rate of *E. coli* expressing a highly specific ThTPase as a GST fusion protein (GST-ThTPase (9)) with bacteria expressing GST alone. In M9 medium containing 10 mM pyruvate, GST bacteria rapidly entered an exponential growth phase and reached a plateau after about 9 h (Fig. 5*A*). In GST-ThTPase bacteria an "intermediate plateau" appeared after 5 h. It lasted about 5–6 h, before exponential growth resumed.

The ThTP content was determined in parallel in GST and GST-ThTPase bacteria (Fig. 5*B*). In GST bacteria, there was an important but transient accumulation of ThTP, reaching a maximum of nearly 400 pmol per mg of protein after 1 h. In contrast, when GST-ThTPase was expressed instead of GST, no significant amount of ThTP was detected (Fig. 5*B*).

It could be argued that GST-ThTPase overexpression induces a slow down in cell growth independently of ThTP hydrolysis. Indeed, because the molecular weight of the fusion protein (50 kDa) is twice higher than that of GST (25 kDa), its synthesis should require more energy. Therefore, we tested the effect of a larger fusion protein, GST-EFHC1 (100 kDa), on bacterial growth. EFHC1, under investigation in our laboratory, is a eukaryotic protein containing one EF-hand. In the presence of this fusion protein, which is irrelevant to thiamine metabolism and devoid of ThTPase activity, bacteria grew at the same rate as GST bacteria (not shown).

When bacteria were grown in M9 medium supplemented with pyruvate in the absence of IPTG, no differences in the growth rate between GST and GST-ThTPase bacteria were observed

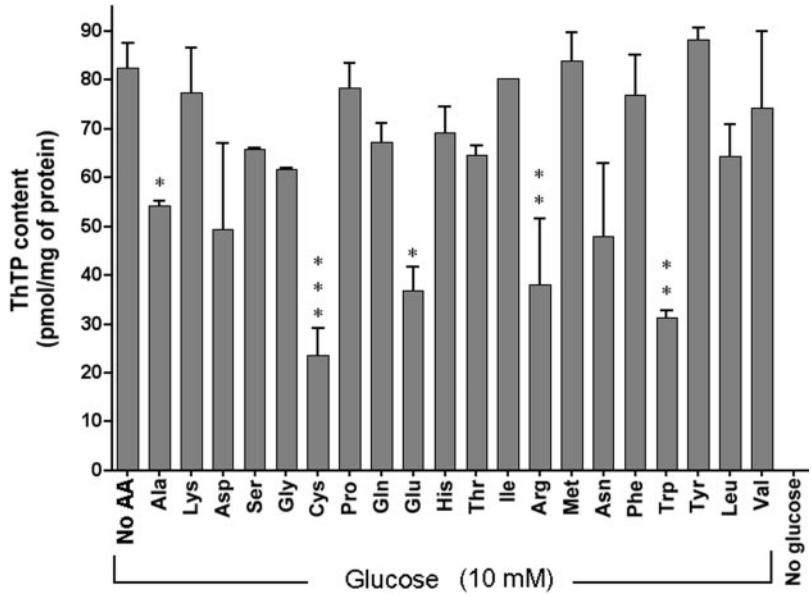


FIG. 4. **Effect of amino acids on the accumulation of ThTP in M9 medium in the presence of 10 mM glucose.** The bacteria were incubated for 30 min in M9 medium supplemented with 10 mM glucose either in the absence (*No AA*) or in the presence of the indicated amino acid (10 mM). Because of low solubility, tyrosine was used at 5 mM. No glucose was present in the last column, and ThTP was undetectable. For the Kruskal-Wallis test, $p < 0.0001$. Dunn's multiple comparisons test was used for comparison with control (***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$). The results are expressed as the mean ± S.D. for 2–8 experiments.

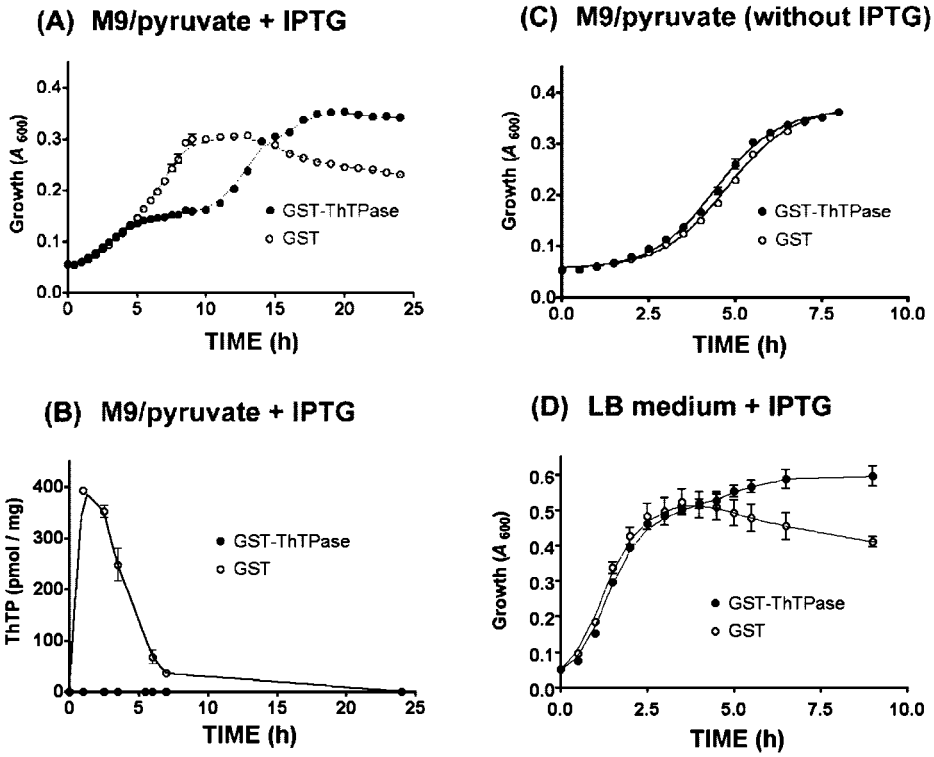


FIG. 5. **Comparison of growth and ThTP content in bacteria overexpressing either GST or GST-ThTPase.** *A*, bacteria grown overnight in LB medium were centrifuged and resuspended in minimal M9 medium. The bacteria were diluted to an $A_{600}$ of 0.05, and the medium was supplemented with 10 mM pyruvate. After 1 h IPTG (0.75 mM) was added. *B*, ThTP was determined in both cultures at the times indicated. *C*, bacteria transferred to M9 medium supplemented with pyruvate in the absence of IPTG. *D*, bacteria transferred to LB medium containing IPTG. All media contained ampicillin (200 μg/ml). The results are expressed as the mean ± S.D. for 3–8 experiments.

(Fig. 5*C*). Under these conditions GST-ThTPase bacteria contain about half the amount of ThTP present in the GST bacteria (not shown). Indeed our previous results have shown that in the absence of IPTG ThTPase activity is about 10% of the activity in the presence of IPTG (9). This activity, although higher than endogenous ThTPase activity in *E. coli*, is not sufficient to hydrolyze all ThTP and, thus, impair bacterial growth.

It could also be argued that the intermediate plateau observed in GST-ThTPase bacteria (Fig. 5*A*) is due to some kind of toxicity of ThTPase moiety independent of ThTP hydrolysis. Although several studies showed the high specificity of the 25-kDa ThTPase for ThTP, a significant hydrolysis of ATP in GST-ThTPase bacteria cannot be excluded. Determination of ATP in GST- and GST-ThTPase- expressing bacteria showed no significant differences (not shown). Moreover, when grown in LB medium supplemented with IPTG (Fig. 5*D*), both GST and GST-ThTPase bacteria rapidly entered the exponential growth phase and reached a stationary phase after about 3 h. In this medium, rich in amino acids, the expression of GST-ThTPase did not cause the appearance of an intermediate plateau. As expected, no ThTP was detected in either of the two cultures (not shown). These results strongly suggest that the intermediate plateau observed in GST-ThTPase bacteria in the absence of amino acids (Fig. 5*A*) is really due to ThTP depletion.

After induction by IPTG, we consistently observed a decrease in the absorbance of the medium containing GST bacteria after a plateau was reached in minimal (Fig. 5*A*) as well as in LB (Fig. 5*D*) medium. After induction the amount of intracellular GST reached 10% of total protein, significantly more than GST-ThTPase (not shown). Protein overexpression is a significant metabolic burden for bacteria (18) and a slow-down of growth after induction of GST is, therefore, not surprising.

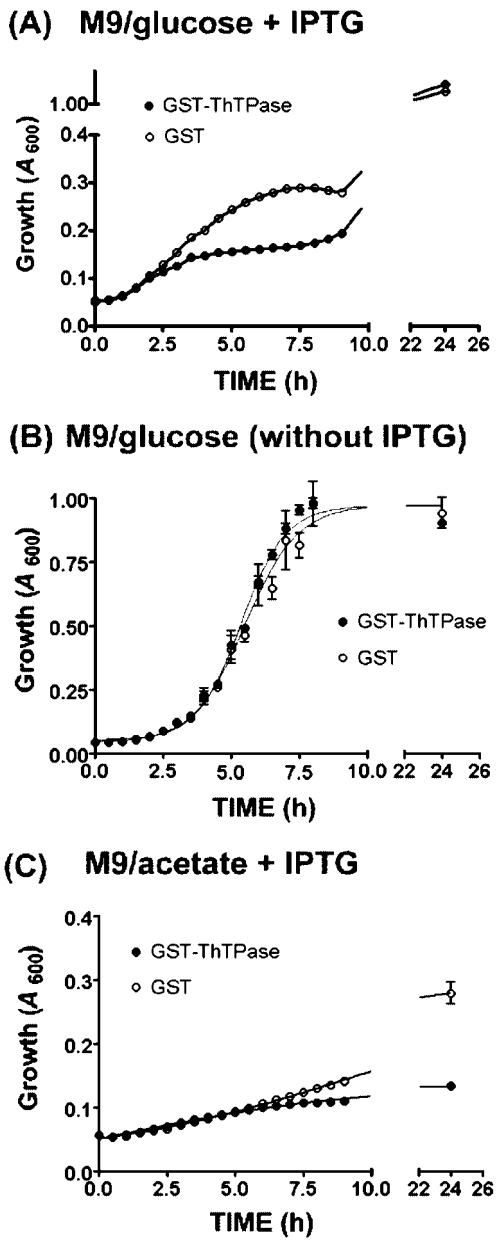


FIG. 6. **Growth curves of *E. coli* in minimal medium supplemented with glucose or acetate.** The bacteria were obtained as described in legend to Fig. 5. Expression of plasmid-encoded protein was induced by IPTG (0.75 mM) after 1 h. In all cases 200 $\mu$g/ml ampicillin was present. *A*, bacteria grown in M9 medium in the presence of 10 mM glucose and induced by IPTG. *B*, bacteria grown in M9 medium in the presence of 10 mM glucose, without induction by IPTG. *C*, bacteria grown in M9 medium in the presence of 10 mM acetate and induced by IPTG. The results are expressed as the mean ± S.D. for at least three experiments.

In the experiment shown in Fig. 5*A*, the differences in growth rate between GST- and GST-ThTPase-expressing bacteria were studied in the presence of pyruvate as the carbon source. We also observed those differences using two other substrates inducing ThTP accumulation, *i.e.* glucose and acetate (Fig. 6). As with pyruvate, an intermediate plateau was observed with bacteria depleted in ThTP but not in the absence of IPTG. In glucose, the growth curves diverged already after 2.5 h (compared with 4.5 in pyruvate), whereas in acetate, the curves diverged only after 6 h. In each case, the growth curves started to diverge when the absorbance doubled, *i.e.* after approximately one division. In glucose, GST-ThTPase bacteria recovered after the intermediate plateau phase, and after 4 h the cell density was the same for both strains. In contrast, GST-ThTPase bacteria grown in acetate showed no recovery of growth after 24 h. In this case, ThTP depletion appears to induce a long-term impairment of growth rather than an intermediate plateau. This inability to recover may be related to the fact that growth in acetate is considerably slower than in glucose. Acetate is indeed a poor energetic substrate (as shown in Table I, intracellular ATP levels are lower in acetate than with most other carbon sources).

## DISCUSSION

Two main findings emerge from the present study, (i) intracellular ThTP levels depend on the nature of the organic carbon source, and (ii) ThTP plays a role in the fast adaptation of bacteria to amino acid starvation. We have defined for the first time the experimental conditions under which a microorganism, *i.e. E. coli*, will accumulate ThTP in appreciable amounts and in a reproducible way. These conditions are (i) switching from rich LB medium to a minimal medium devoid of amino acids and (ii) the presence of an appropriate carbon source such as glucose or pyruvate. Under these conditions there is a fast and important increase in ThTP levels (up to 20% of total thiamine, corresponding to an intracellular concentration of at least 20 $\mu$M). The phenomenon is transient and after 1–3 h, the cellular ThTP content markedly decreases. This rapid decrease can only be explained by hydrolysis. ThTPases have indeed been shown to exist in bacteria (13), but they seem to be unrelated to the mammalian 25-kDa ThTPase.

An important observation is that ThTP accumulation is impaired when the minimal medium, containing glucose, is supplemented with amino acids (*i.e.* a mixture of the 20 amino acids at 0.5 mM each). Taken together, those data support the idea that the appearance of ThTP is a signal involved in the adaptation of the bacteria to stringent conditions, such as amino acid starvation.

It should be emphasized that ThTP levels reached were similar (about 300 pmol/mg of protein) whether the bacteria were in the stationary (Fig. 1) or the exponential (Fig. 5*A*) growth phase. Thus, the important factor for ThTP accumulation in minimal medium is not the physiological state of the culture but the nature of the carbon source.

The second finding points more directly to the physiological role of ThTP, as we have shown that *E. coli* rendered ThTP-deficient by overexpression of a specific ThTPase exhibit an intermediate plateau of growth when they are switched to the minimal medium under conditions that would normally lead to ThTP accumulation. This does not mean that ThTP is always required for optimal growth, because we have seen that bacteria grown in optimal LB medium contain no ThTP. Rather, ThTP appears to be required temporarily when the bacteria have to adapt to a medium that contains no amino acids. ThTP accumulation may be only an early step in a cascade of adaptive biochemical processes.

Although the present study appears as a useful basis for understanding the biological role of ThTP in bacteria, it leaves a number of important questions unanswered. First, we know very little about the enzymatic mechanism of ThTP synthesis and about the biochemical events that regulate ThTP concentrations in the cell. Yet some indications can be drawn from our results. We have seen that when the bacteria accumulate a certain amount of ThTP they loose an equimolar amount of ThDP, suggesting that the diphosphate is the precursor. A phosphate donor should thus be required, and the most obvious candidate is ATP. However, the data of Table I show only a poor correlation between the cellular ATP content and the amount of ThTP appearing in the cells. It is particularly noteworthy that malate and oxaloacetate, which increase cellular ATP, are

completely ineffective in increasing ThTP, whereas acetate, which is a very poor substrate for ATP production, induces an appreciable accumulation of ThTP. Therefore, if we assume that ATP is in fact the direct phosphate donor, some other factor(s) probably controls the rate of synthesis or hydrolysis. As we pointed out, substrates that can be processed to form acetyl-CoA (or succinyl-CoA) raise ThTP levels, whereas oxaloacetate, which reacts with acetyl-CoA to form citrate, is unable to induce ThTP accumulation. It is, thus, tempting to speculate that acetyl-CoA (and possibly other thioesters) is either an activator of ThTP synthesis or an inhibitor of its hydrolysis. Conversely, the presence of amino acids would inhibit ThTP synthesis or stimulate its hydrolysis.

A second important and unanswered question is, once ThTP is present, what are its cellular targets? Preliminary experiments have shown that [$\gamma$-$^{32}$P]ThTP phosphorylates a 26-kDa protein in *E. coli* (not shown). It will be important to identify this protein to find out whether this phosphorylation is biologically relevant. Alternatively, the target may be a receptor protein binding ThTP, as is observed for cAMP and transcription factors in bacteria (19). Even mechanisms involving a riboswitch (20) may be considered.

It is interesting to compare ThTP with another molecule that attracted much interest during recent years, *i.e.* polyP. Like ThTP, polyP transiently accumulates when bacteria are switched from a rich to a minimal medium (21). This accumulation is stimulated by a rapid rise in levels of guanosine pentaphosphate (22), which strongly inhibits exopolyphosphatase (8). PolyP in turn promotes protein degradation by the Lon protease (17), supplying amino acids needed for growth. Bacteria deficient in polyP kinase and, thus, unable to synthesize polyP exhibit an important lag in growth that is reversed in the presence of amino acids (16). In this case, however, the growth lag is observed immediately after starting the experiment, whereas in our experiments there was a delay of 3–5 h between induction of GST-ThTPase and the slow-down of growth corresponding to the intermediate plateau (Figs. 5*A* and 6, *A* and *C*). A possible explanation would be that, even in the complete absence of ThTP, a relatively constant growth rate can be maintained as long as the intracellular concentration of some key intermediate(s) (may be amino acids) is maintained above a certain threshold. Once the intermediates are exhausted, the growth slows down, leading to an intermediate plateau. ThTP would be part of a rescue system stimulating the synthesis of the intermediate(s), thus facilitating bacterial growth. The observation that with three different substrates (glucose, pyruvate, and acetate) the intermediate plateau starts after different time intervals (respectively, 2.5, 4.5, and 6 h) but always approximately after doubling of the cell density would be in favor of the exhaustion of a key intermediate in metabolism.

Further work will be required to substantiate this interpretation, but our results indicate at least that ThTP levels in *E. coli* do not directly control the growth rate. Rather, the initial accumulation of ThTP appears as a transient signal inducing a cascade of secondary biochemical processes, eventually resulting in growth facilitation. Although most of our results were obtained with the BL21 strain deficient in Lon protease, the adaptive mechanism involving ThTP accumulation probably exists in wild-type *E. coli* as well, as we have shown a similar initial accumulation of ThTP using the K-12 strain. On the other hand, ThTP accumulation requires a suitable carbon source, whereas there is no indication that the nature of the carbon source is important for polyP synthesis. Therefore, no obvious relationship appears to exist between the two responses to amino acid starvation.

Even if the two mechanisms are unrelated, we should emphasize some striking similarities between ThTP and polyP. Both compounds are found in most organisms ranging from bacteria to mammals. Both compounds contain high energy phosphoanhydride bonds and are increased in bacteria after a stress such as amino acid starvation. Their role in eukaryotes remains enigmatic, but ThTP may phosphorylate certain proteins (2), whereas polyP stimulates mammalian protein kinase target of rapamycin, involved in the proliferation of mammary cancer cells (23). Both compounds are conserved through evolution and appear to be involved in basic cellular processes.

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