The mtrAB Operon of Bacillus subtilis Encodes GTP Cyclohydrolase I (MtrA), an Enzyme Involved in Folic Acid Biosynthesis, and MtrB, a Regulator of Tryptophan Biosynthesis

PAUL BABITZKE, 1 PAUL GOLLNICK, 2 AND CHARLES YANOFSKY 1*

Department of Biological Sciences, Stanford University, Stanford, California 94305, 1 and Department of Biological Sciences, State University of New York at Buffalo, Buffalo, New York 14260 2

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mtrA of Bacillus subtilis was shown to be the structural gene for GTP cyclohydrolase I, an enzyme essential for folic acid biosynthesis. mtrA is the first gene in a bicistronic operon that includes mtrB, a gene involved in transcriptional attenuation control of the trp genes. mtrA of B. subtilis encodes a 20-kDa polypeptide that is 50% identical to rat GTP cyclohydrolase I. Increased GTP cyclohydrolase I activity was readily detected in crude extracts of B. subtilis and Escherichia coli in which MtrA was overproduced. Biochemical evidence indicating that MtrA catalyzes dihydronoeopterin triphosphate and formic acid formation from guanosine triphosphate is presented. It was also shown that mtrB of B. subtilis encodes a 6-kDa polypeptide. Expression of mtrB is sufficient for transcriptional attenuation control of the B. subtilis trp gene cluster in Escherichia coli. Known interrelationships between genes involved in folic acid and aromatic acid biosynthesis in B. subtilis are described.

GTP cyclohydrolase I from a variety of organisms catalyzes dihydronoeopterin triphosphate and formic acid formation from GTP. This reaction is the first step in the biosynthetic pathway leading to synthesis of the pteridine portion of folic acid in prokaryotes (4, 40), tetrahydrobiopterin in mammals (5, 12, 32), and pteridine-containing pigments in insects (29, 39). GTP cyclohydrolase I has been identified in several bacterial species, including Escherichia coli (40), Salmonella typhimurium (7), Serratia indica (22), Lactobacillus plantarum (19), Pseudomonas cocovenanans (27), Pseudomonas sp. (6), and Bacillus stearotherophilus (36). Despite the numerous prokaryotic and eukaryotic species in which GTP cyclohydrolase I has been identified, the gene that putatively encodes this enzyme has been isolated only from E. coli (21), a rat (13), and Drosophila melanogaster (29).

The mtr (methyltryptophan resistance) locus of Bacillus subtilis was identified initially as the regulatory locus for the trp gene cluster of this organism (16). The term gene cluster is used in place of the term operon when referring to trpEDCFBA of B. subtilis because these genes are known to reside within a supraoperon concerned with aromatic amino acid and histidine biosynthesis (14, 15). The product of mtr is thought to be a trans-acting RNA-binding regulatory protein that is activated by tryptophan (34). Transcription termination at the attenuator preceding the trp gene cluster presumably occurs as a consequence of binding of the activated mtr-encoded protein to the nascent transcript (23, 34). The mtr locus has been cloned and sequenced and found to be a two-gene operon with the capacity to encode polypeptides of 22 (MtrA) and 8 (MtrB) kDa (8). MtrB has some sequence similarity to RegA, an RNA-binding regulatory protein of bacteriophage T4 (38). Previous searches of the available databases did not reveal any proteins with significant homology to MtrA (8). However, J. O’Donnell, University of Alabama, recently informed us that B. subtilis MtrA is homologous to the recently published GTP cyclohydrolase I sequence from a rat (13) and to GTP cyclohydrolase I from D. melanogaster (31). Comparison of the predicted amino acid sequence of MtrA with that of rat GTP cyclohydrolase I revealed that they were 50% identical. On the basis of this homology, we performed experiments to determine whether MtrA of B. subtilis is GTP cyclohydrolase I. Here we describe results which established that MtrA is GTP cyclohydrolase I. We also demonstrate that MtrB is sufficient for regulation of expression of the trp gene cluster of B. subtilis in E. coli.

MATERIALS AND METHODS

Chemicals and enzymes. Neopterin and rifampin were purchased from Sigma Chemical Co. GTP was obtained from Pharmacia. Taq polymerase was purchased from Perkin-Elmer/Cetus and used in accordance with the manufacturer’s specifications. Restriction enzymes and T4 DNA ligase were from Bethesda Research Laboratories. Mung bean nuclease was obtained from New England BioLabs. Prestained low-molecular-weight protein standards were from Gibco-BRL.

Bacterial strains and plasmids. All of the bacterial strains used in this study are described in Table 1. B. subtilis CYBS13 was constructed by integration of a segment of ptrpBG1 that contains this translational fusion of trpE of B. subtilis and lacZ of E. coli (33) into the amyE locus of strain 1A62 by selecting for chloramphenicol resistance. Strain CYBS17 was constructed by transforming CYBS13 with chromosomal DNA prepared from strain JH584 (aroB), trp* transformants were screened for aroB (shikimic acid auxotrophy).

Plasmids pTZ18R (United States Biochemical Co.), pPG1-2 (37), pHY300PLK (18), pSI45 (8), pTTmtrAB (8), pTTmtrB (8), and ptrpE-lacZ (8) have already been described. Plasmid ptrpE-lacZ was constructed by linearization of ptrpE-lacZ at the PsiI site in the ampicillin resistance gene, removal of the sticky ends with mung bean nuclease,
and religation. pTZmtrAB was constructed by subcloning the 1,150-bp EcoRI-HindIII fragment containing mtrAB from pTTmtrAB (8) into the EcoRI-HindIII sites of pTZ18R. pTZmtrB was constructed by subcloning the 580-bp BamHI-HindIII fragment containing mtrB from pTTmtrB (8) into the BamHI-HindIII sites of pTZ18R. pTZmtrA was constructed by using the polymerase chain reaction to amplify the mtrA region from genomic DNA. The oligonucleotides used as primers in the reaction were designed such that an EcoRI site was created upstream of mtrA at position 371 (8) and a SaII site was created downstream of mtrA at position 1022 (8). The 655-bp fragment that was produced was digested with EcoRI and SaII and ligated into the EcoRI-SaII sites of pTZ18R. The polymerase chain reaction was carried out by using an Ericonp Thermocycler. Oligonucleotides were synthesized by using an Applied Biosystems 380B DNA synthesizer. Plasmids pTZmtrAB, pTZmtrA, and pTZmtrB were constructed such that they retained the natural mtrA and/or mtrB ribosome-binding sites but not the endogenous mtrP promoter.

**Transformation procedures.** E. coli cells were transformed by the method of Kushner (25). B. subtilis was transformed by using natural competence (1). Appropriate antibiotics were added, as needed, to the following concentrations: kanamycin, 50 μg/ml; ampicillin, 100 μg/ml; chloramphenicol, 20 μg/ml; tetracycline, 20 μg/ml.

**DNA isolation.** Plasmid DNA was isolated by the alkaline lysis procedure of Birnboim and Doly (3) for CsCl-purified preparations and by a modified method of the form of Ish-Horowicz and Burke (17) for miniscreens. Chromosomal DNA was prepared from B. subtilis as previously described (28).

**Cell growth and preparation of crude extracts.** Growth of E. coli cells used in the preparation of crude extracts was done as previously described (37). The cells from each 200-ml culture were harvested, washed, and suspended in 5 ml of lysis buffer (50 mM Tris-HCl [pH 8.5], 1 mM dithiothreitol). Cells were lysed by sonication at 30 W with a Heat Systems-Ultrasonics W-225R sonicator. Cell debris was removed by centrifugation for 15 min in a microcentrifuge. Protein concentrations were determined by the Bio-Rad protein assay.

**B. subtilis** cells were grown in Luria broth to the late log phase (Klett 110, green filter no. 54), at which time a 200-ml culture volume was harvested, washed, and suspended in 1.5 ml of lysis buffer. Cell lysis and protein determinations were done as described above.

**Enzyme assays.** GTP cyclohydrolase I activity was determined by the fluorescence method, essentially as described by Yim and Brown (40). This assay measures dihydronor- terin triphosphate formation from GTP. Standard 0.5-ml reaction mixtures contained 100 mM Tris-HCl (pH 8.5), 2.5 mM EDTA (adjusted to pH 8.0 with KOH), 1 mM dithiothreitol, 1 mM GTP, and protein extract. Incubation was at 42°C. At 30 min after the start of the reaction, an equal volume of activated charcoal (40 mg/ml) was added to adsorb both dihydronorpterin triphosphate and unreacted GTP. The mixture was filtered through a Millipore filter (type HA, 0.45 μm). The filter was then washed with 5 ml of water, 5 ml of 5% ethanol, and 5 ml of 50% ethanol that also contained 3.1% NaOH. During the adsorption and elution from the charcoal, dihydronorpterin triphosphate is quantitatively oxidized to neopterin triphosphate (40). The final wash was collected and analyzed for neopterin triphosphate by fluorescence (265-nm excitation, 450-nm emission) with a Gilford Fluoro IV spectrophluorometer. Neopterin at 25 μM was used as the standard.

β-Galactosidase assays were performed as described by Miller (30). Cells used in the assay were grown in minimal medium supplemented with 1% casein hydrolysate in the absence or presence of tryptophan (100 μg/ml).

**RESULTS**

**Homology between MtrA and rat GTP cyclohydrolase I.** As reported previously (8), the mtrA nucleotide sequence from B. subtilis contains an open reading frame that can encode a 190-residue polypeptide. Initial searches of the available protein data bases did not disclose any protein with significant homology to MtrA (8). A subsequent search of the updated GenBank/EMBL data bank performed by J. O’Donnell, University of Alabama (31), revealed extensive homology between a predicted Drosophila protein, Mtra, and rat GTP cyclohydrolase I (13). It can be seen in Fig. 1 that MtrA

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**TABLE 1. Bacterial strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
<td>B. subtilis 1A62</td>
<td>trpA5</td>
<td>This study</td>
</tr>
<tr>
<td>B. subtilis CYBS13</td>
<td>trpA5 amyE::[trpp-(trpE'--lacZ)]</td>
<td>This study</td>
</tr>
<tr>
<td>B. subtilis CYBS17</td>
<td>araB amyE::[trpp-(trpE'--lacZ)]</td>
<td>This study</td>
</tr>
<tr>
<td>B. subtilis CYBS200</td>
<td>CYBS17/pHY300PLK (Te')</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli JH584</td>
<td>araB</td>
<td>J. A. Hoch†</td>
</tr>
<tr>
<td>E. coli JM109 (DE3)</td>
<td>endA1 recA1 gyrA96 thi hsdR17 (r− m−) relA1 supE44 Δ(lac-proAB) F' traD36 proAB lacIq ZAM15 (DE3)</td>
<td>Promega</td>
</tr>
</tbody>
</table>

* BOSC, Bacillus Genetic Stock Center, Department of Biochemistry, Ohio State University, Columbus.
† Department of Microbiology, Scripps Clinic and Research Foundation, La Jolla, Calif.
and the rat protein are 50% identical. When the comparison is extended to include conservative amino acid differences, the proteins are 65% similar. These comparisons suggested that MtrA was GTP cyclohydrolase 1 of \textit{B. subtilis}.


g Identification of MtrA as GTP cyclohydrolase 1. Our initial strategy to determine whether MtrA has GTP cyclohydrolase 1 activity was to overexpress the \textit{B. subtilis} \textit{mtr} operon in \textit{E. coli}. We used the T7 expression system described by Tabor and Richardson (37). Recombinant plasmid pTZ mtrAB and control plasmid pTZ18R were transformed into \textit{E. coli} K38/pGP1-2. By using this system, we produced MtrA and MtrB to approximately 30% of total \textit{E. coli} protein (Fig. 2). The 20- and 6-kDa polypeptides that were observed are in excellent agreement with the predicted molecular masses of MtrA (22 kDa) and MtrB (8 kDa), respectively (Fig. 2, lane 2). Neither of these polypeptides was present in the extract from the control strain (Fig. 2, lane 1). These extracts were then assayed for GTP cyclohydrolase I activity by using the procedure of Yim and Brown (40). Overproduction of MtrA and MtrB resulted in a 66-fold increase in the level of GTP cyclohydrolase I activity (Table 2).

To determine whether MtrA was solely responsible for the observed increase in GTP cyclohydrolase I activity, plasmid pTZmtrA was introduced into strain K38/pGP1-2. This plasmid is essentially identical to pTZmtrAB, except that \textit{mtrB}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
Strain & Plasmid & Activity (U)a & Sp act (U/mg of protein) \\
\hline
CY15250 & pTZ18R & 1 & 10 \\
CY15251 & pTZmtrAB & 66 & 660 \\
CY15252 & pTZmtrA & 40 & 400 \\
CYB200 & pHY300PLK & 8 & 8 \\
CYB201 & pSI45 (\textit{mtr}AB) & 94 & 94 \\
\hline
\end{tabular}
\caption{Assay of extracts of various strains for GTP cyclohydrolase I activity}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
Plasmids present & Relative \beta-galactosidase activity\textsuperscript{a} & \\
\hline
& No tryptophan & With tryptophan \\
\hline
ptrpE-lacZZ + pTZ18R & 1.00 & 0.77 \\
ptrpE-lacZZ + pTZmtrAB & 0.60 & 0.10 \\
ptrpE-lacZZ + pTZmtrB & 0.58 & 0.04 \\
ptrpE-lacZZ + pTZmtrA & 0.75 & 0.50 \\
\hline
\end{tabular}
\caption{MtrB regulation of attenuation in the \textit{B. subtilis} \textit{trp} leader region}
\end{table}

\textsuperscript{a} The values shown are averages of four experiments. The value obtained with ptrpE-lacZZ plus pTZ18R without tryptophan was set at 1.00.

has been deleted, pTZmtrA clearly directed overexpression of only \textit{mtrA} in K38/pGP1-2 (Fig. 2, lane 3). This extract was also assayed for GTP cyclohydrolase I activity. A 40-fold increase in enzyme activity was observed (Table 2), which was comparable to that obtained with the extract containing both MtrA and MtrB. Divalent cations were apparently not required for activity, as the enzyme was equally active in the presence of 25 mM EDTA (data not shown).

In addition to the experiments performed by using overexpressing strains of \textit{E. coli}, it was also of interest to determine whether GTP cyclohydrolase I activity could be increased by overexpressing \textit{mtrA} in \textit{B. subtilis}. Strain CYB217 was transformed with plasmid pSI45, which carries \textit{mtrAB} under control of its natural promoter. The same strain was also transformed with vector pHY300PLK as a control. An increased level of MtrA was observed in the strain that contained pSI45 relative to the control strain, in which \textit{mtrA} was present in a single copy (Fig. 2, lanes 4 and 5). These extracts were assayed for GTP cyclohydrolase I activity, and the extract from the strain harboring pSI45 showed a 12-fold increase in enzyme activity compared with the control strain (Table 2). Interestingly, a low but reproducible level of enzyme activity was detected in the control strain containing a single copy of \textit{mtrA}. Taken together, these results demonstrate that \textit{mtrA} encodes GTP cyclohydrolase I from \textit{B. subtilis}.

\textit{MtrB} is sufficient for regulation of expression of the \textit{trp} genes of \textit{B. subtilis} in \textit{E. coli}. In a previous study, plasmids containing \textit{mtrB}, or \textit{mtrA} and \textit{mtrB}, were introduced into an \textit{E. coli} strain containing a plasmid having a fusion of the \textit{B. subtilis} \textit{trp} regulatory region to \textit{E. coli} lacZ as a \textit{trpE'-lacZ} gene fusion. In the presence of tryptophan, the plasmid containing both \textit{mtrA} and \textit{mtrB} conferred regulation of \beta-galactosidase synthesis, whereas the plasmid containing \textit{mtrB} did not (8). In view of the finding reported here, that \textit{mtrA} encodes GTP cyclohydrolase I, the above-described experiment was repeated by using more efficient expression plasmids to test the possibility that the \textit{mtrB}-expressing plasmid used previously did not direct the synthesis of sufficient MtrB protein to be effective. Accordingly, the above-described experiment was performed with plasmids in which \textit{mtrB} (pTZmtrB), \textit{mtrA} (pTZmtrA), or \textit{mtrA} and \textit{mtrB} (pTZmtrAB) were expressed from a T7 promoter in strain JM109(DE3), which produces T7 RNA polymerase. The presence of either plasmid containing \textit{mtrB} permitted tryptophan regulation of expression of the \textit{B. subtilis} \textit{trpE'-lacZ} fusion (Table 3). Expression of \textit{mtrA} alone did not allow tryptophan regulation of \beta-galactosidase production from ptrpE-lacZ2 (Table 3). These findings demonstrate that \textit{mtrB} is sufficient for regulation of transcription termination in the leader region preceding the \textit{trp} genes of \textit{B. subtilis}. 

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of cell extracts from the following strains: lane 1, CY15250(pTZ18R); lane 2, CY15251(pTZmtrAB); lane 3, CY15252 (pTZmtrA); lane 4, CYB200(pHY300PLK); lane 5, CYB201 (pSI45) \textit{mtr}AB. A 25-\(\mu\)g protein sample was loaded in lanes 1 to 3, and a 100-\(\mu\)g sample was used in lanes 4 and 5. Locations of prestained low-molecular-weight protein standards are indicated in kilodaltons.
Folic acid biosynthesis in prokaryotes is a multistep process requiring formation of a pteridine ring and subsequent addition of p-aminobenzoic acid and glutamic acid residues. Folic acid, in turn, is involved in one carbon metabolism in the synthesis of methionine, glycine, thymine, purines, and pantothenic acid (11). We have identified mtrA of B. subtilis as the gene that encodes GTP cyclohydrolase I. This enzyme catalyzes formation of dihydroneopterin triphosphate by removal of carbon 8 from GTP as formic acid. This reaction is the first step in the synthesis of the pterin moiety that, upon further modification, is incorporated into folic acid (40).

We directed the synthesis of appreciable levels of GTP cyclohydrolase I in E. coli and B. subtilis by overexpressing the B. subtilis mtrAB operon. Overexpression of mtrAB, and mtrA alone, allowed us to establish that mtrA encodes a 20-kDa polypeptide (Fig. 2) that is sufficient for GTP cyclohydrolase I activity (Table 2). In addition, we found that the enzyme does not require divalent cations for activity. The absence of a divalent-cation requirement for GTP cyclohydrolase I activity has been observed in all of the organisms in which this enzyme has been characterized (4–7, 12, 19, 22, 27, 32, 36, 39, 40).

Comparison of the amino acid sequence of MtrA with that of the rat enzyme revealed striking homology. The two enzymes are 50% identical, and homology extends throughout MtrA (Fig. 1). This degree of homology between enzymes from organisms as unrelated as B. subtilis and rats suggests that strong selective pressure to maintain the overall structure of GTP cyclohydrolase I exists.

No prokaryotic strain deficient in GTP cyclohydrolase I has been described. In a previous study, Gollnick et al. (8) reported the inability to construct a strain in which mtrA was disrupted or deleted. However mtrB was dispensable. They reasoned that mtrA may serve some essential function in B. subtilis. The finding that mtrA of B. subtilis encodes GTP cyclohydrolase I is consistent with this explanation. The essential nature of MtrA may indicate that one or more of the one-carbon metabolites that are products of folate-dependent pathways are limiting in B. subtilis. Alternatively, a branch point leading to the synthesis of some, as yet unidentified, essential pteridine derivative may exist. This second possibility is consistent with the isolation of several pteridine compounds from bacteria that are not involved in folate acid synthesis. Examples are the pteridine cofactors of alcohol dehydrogenase (2) and phenylalanine hydroxylase (10) of Pseudomonas species. In addition, the azaperidine antibiotic toxoflavin has been isolated from P. cocovenenans (27). The roles played by various pterins in B. subtilis have not been established.

The findings described in this report reveal an interesting interrelationship between folic acid and tryptophan biosynthesis in B. subtilis (Fig. 3). Transcription of the trp gene cluster (trpEDCFBA) is regulated in response to the availability of tryptophan (34). It is thought that a trans-acting regulatory factor encoded by the mtr locus recognizes a specific 10-base nucleotide sequence in the leader transcript, causing transcription termination (24). In addition, binding of this protein to nonterminated trp transcripts may inhibit translation by favoring a transcript secondary structure that sequesters the trpE ribosome-binding site (24). Inhibition of translation of the unlinked trpG coding region is also believed to be mediated by an mtr product (23). In a previous study, evidence was presented suggesting that both MtrA and MtrB are necessary for attenuation control of the B. subtilis trp gene cluster (8). The findings that mtrA encodes GTP cyclohydrolase I and that MtrB is sufficient for trp attenuation regulation indicate that MtrB is the trans-acting regulator of the B. subtilis trp gene cluster and that MtrA is not involved in this process.

Another gene cluster that links folic acid formation and tryptophan biosynthesis in B. subtilis is the folic acid operon described by Slocok et al. (35). This operon includes pab, trpG, pabC, and sul, as well as two or more additional open reading frames, pab and pabC are involved in p-aminobenzoic acid synthesis, while trpG encodes an amphiphilic polypeptide that is required for synthesis of both p-aminobenzoic acid and tryptophan (35). The sul gene encodes dihydropteroate synthase, the enzyme responsible for condensation of p-aminobenzoic acid and the pteridine ring (Fig. 3). In an analysis of the trpG nucleotide sequence (35), a nine-base sequence (AGATGAGGT) that overlaps the trpG ribosome-binding site was found to have eight nucleotides in common with the regulatory sequence (GAATGAGTT) thought to be involved in trp attenuation and translational repression of the trpEDCFBA gene cluster (24, 34). Slocok et al. (35) postulated that the product of the mtr locus may bind to this nine-base sequence, which overlaps the trpG ribosome-binding site, thereby blocking translation initiation (Fig. 3). This interpretation is supported by the observation that synthesis of TrpG is negatively regulated by tryptophan and mtr in vivo (20) and by analysis of trpG expression using a trpG'-lacZ fusion (23).

It is important to note that the aroF, aroB, and aroH genes lie immediately upstream and the hisF, tyrA, and aroE genes lie immediately downstream of the trpEDCFBA gene cluster, in what has been termed a superoperon involved in aromatic
amino acid and histidine biosynthesis (14, 15) (Fig. 3). Transcripts that presumably originate from the aroFBH gene cluster enter the trp leader region (34). Since the stop codon of aroH is located in the trp promoter (9), the trp attenuator is the initial transcription terminator for the aroFBH gene segment. Thus, the mtr and folate operons, as well as the aromatic amino acid suprasoperon, contain genes involved in the biosynthesis of both folic acid and tryptophan. (Fig. 3). These gene arrangements appear to facilitate coordinate regulation of the biosynthesis of folic acid, the aromatic amino acids, and histidine in B. subtilis.

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