Characterization of *Methanobacterium thermoautotrophicum* Marburg Mutants Defective in Regulation of \(\ell\)-Tryptophan Biosynthesis

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Three nitrosoguanidine-induced mutants of the archaeon *Methanobacterium thermoautotrophicum* Marburg resistant to \(\ell\)-methyltryptophan were isolated and characterized. They were found to take up \(\ell\)-tryptophan, as wild-type cells, via an energy-dependent, low-affinity transport system specific for \(\ell\)-tryptophan, with a \(K_m\) of 300 \(\mu\)M and a \(V_{\text{max}}\) of 7 nmol/mg (dry weight)/min. Resistance to \(\ell\)-methyltryptophan was not due to feedback-resistant anthranilate synthase but to constitutive expression of the *trp* genes, as measured by the specific activities of anthranilate synthase and tryptophan synthase, the enzymes encoded by *trpEG* and *trpB*, respectively, of the *trpEGCFBAD* gene cluster. Estimation of *trpE* mRNA obtained from mutant cells grown in minimal medium with or without \(\ell\)-tryptophan suggested that constitutive expression resulted from deficient transcriptional regulation. The enhanced expression of the *trp* genes in the mutants was found to result in intracellular \(\ell\)-tryptophan pools that were two- to fourfold higher than in the wild type. Sequencing of the region upstream of *trpE* revealed in two mutants point mutations mapping on the \(5'\)-side of the archaeal box A, whereas in the third mutant this region did not differ from that of the wild type. These results suggest that (i) in *M. thermoautotrophicum* the \(\ell\)-methyltryptophan-resistant phenotype arises from lesions in components of a regulatory system controlling transcription of the *trp* genes and (ii) cis-acting sequence elements in front of the *trpE* promoter may form part of this system.

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Regulation of tryptophan biosynthesis is a paradigm of gene regulation in different organisms, ranging from the *Bacteria Escherichia coli* (34) and *Bacillus subtilis* (1, 13) to the eucaryon *Saccharomyces cerevisiae* (15). Much less is known about tryptophan regulation in the *Archaea*, even though the complete nucleotide sequences of all *trp* biosynthetic genes are available for *Methanobacterium thermoautotrophicum* (26), *Halofex volcanii* (21, 22), and *Methanococcus jannaschii* (4).

The synthesis of \(\ell\)-tryptophan in methanogens, as supported by \(^{13}\)C labeling experiments and enzymatic analyses (11, 19), is along the pathway described for *Bacteria*. Furthermore, *M. thermoautotrophicum* Marburg, for which a number of mutants are available, including auxotrophic mutants for the amino acid tryptophan (20), is one of the model systems used for gene regulation studies of *Archaea* (3, 27). The *trp* genes of *M. thermoautotrophicum* are organized in an operon-like *trpEGCFBAD* cluster, as found in *Bacteria*, whereas those of *H. volcanii* are organized in two separate operons, *trpCBA* and *trpEGD*, and those of *Methanococcus jannaschii* are scattered over the chromosome in at least four operons.

Regulation of *trp* gene expression has been so far investigated only in *M. thermoautotrophicum*, and we have shown that in this organism those genes are mainly regulated at the transcriptional level (11). The 421-bp region in front of the *M. thermoautotrophicum* *trp* genes contains a sequence homologous with the tryptophan operator site of *E. coli*, suggesting that regulation might also be effected by a repressor in *M. thermoautotrophicum* (26). However, in contrast to *E. coli* and *B. subtilis*, neither a region encoding a leader peptide (35) nor repetitive trinucleotides (GAG, UAG) recognized by the cis-acting regulator MtrB (1) could be found, indicating that *trp* genes in *M. thermoautotrophicum* are probably not regulated by attenuation.

To learn more about regulation of *trp* gene expression in *M. thermoautotrophicum*, we have now isolated mutants resistant to the l-tryptophan analog 5-methyltryptophan. In *Eucarya* and especially *Bacteria*, this selection produced regulatory mutants showing constitutive expression of the *trp* biosynthetic genes (2, 6, 16). In *B. subtilis*, mutants resistant to 5-methyltryptophan could be grouped in five classes (16): (i) those with mutations in an l-Trp uptake system; (ii) mutants with feedback-insensitive anthranilate synthase; (iii) operator-constitutive mutants; (iv) those with mutations in a regulator; and (v) mutants with altered tryptophanyl-tRNA synthetase. In *Archaea*, mutants resistant to amino acid analogs like the histidine analog 1,2,4-triazole-3-alanine (31) or 5-methyltryptophan (12) have been previously reported for *Methanococcus volutae*, but in neither case have the mutations been characterized at the molecular level. Here we describe the characterization of three independent 5-methyltryptophan-resistant mutants of *M. thermoautotrophicum* and discuss possible mechanisms linking the mutations to constitutive expression of *trp* genes.

MATERIALS AND METHODS

**Bacterial strains and oligonucleotides.** Methanogenic strains used were *Methanobacterium thermoautotrophicum* Marburg MBT1 (DSM 2133) (10), the tryptophan-auxotrophic mutant MBT2 (trp-30) (25), as well as the three 5-methyltryptophan-resistant mutants of MBT1: MWR1, MWR4, and MWR8. Oligonucleotides JED2 (5′-AGAAAGTAAGTGGACCTATCATCAGAATTACT-3′) and DGS (5′-CCGCCCTCTCATTCCCAAACCCAC-3′) were used for amplification by PCR of the *trpE* upstream region in MBT1 and the three MWR mutants. *E. coli* DH5α (14) was used for propagation of derivatives of the cloning vector pBlueScript SKII(+) (*Amp′ lacZ) ColE1 repl. con.) (Strategene, La Jolla, Calif.). *E. coli* strains were grown in LB medium (29), with addition of 150 \(\mu\)g of ampicillin per.
ml when needed. Restriction endonuclease digestions were carried out as described by the enzyme manufacturer (MBI/Fermentas, Vilnius, Lithuania), and transformation of E. coli was done according to Sambrook et al. (29).

Growth of *M. thermoautotrophicum*. *M. thermoautotrophicum* MBT1 and mutants were grown at 60°C in 500-ml serum flasks containing 150 to 300 ml of minimal medium (30) supplemented with 8 mM Na$_2$WO$_4$ and 5.6 mM Na$_2$SeO$_3$ (Fluka, Buchs, Switzerland). The flasks were gassed with 2 bars of H$_2$–CO$_2$ (80%:20% [vol/vol]). For growth of the tryptophan-auxotrophic mutant MBT2, the medium was supplemented with 5 mM L-Trp (Fluka). 5-Methyltryptophan-resistant mutants were routinely cultivated in minimal medium supplemented with 7 mM 5-methyl-DL-tryptophan.

Mutagenesis and isolation of 5-methyltryptophan-resistant mutants of *M. thermoautotrophicum*. Mutagenesis with the alkylating agent N-methyl-N-nitro-N-nitrosoguanidine was performed according to Kien et al. (20) and was followed by a selection on minimal medium agar plates containing 3.5, 7, or 14 mM 5-methyl-DL-tryptophan. After 1 week of incubation in a pressure jar under 2 bars of H$_2$–CO$_2$ (80%:20% [vol/vol]), single colonies were picked and transferred to liquid minimal medium containing the same concentration of 5-methyltryptophan as that in the medium from which they were isolated. Mutants spontaneously resistant to 5-methyltryptophan were not observed.

Isolation of total RNA and chromosomal DNA from *M. thermoautotrophicum*. Total RNA was prepared as described previously (11) from cells that had been ruptured after freezing with liquid nitrogen. The liquid nitrogen technique was also used for the isolation and subsequent purification of chromosomal DNA by standard techniques (29).

PCR amplification of the *trpE* upstream region of wild-type and 5-methyltryptophan-resistant mutants. By using the primers DG5 and JE24, the 523-bp *trpE* upstream region extending from nucleotides 47 to 570 of the published sequence (16), which contains 403 nucleotides in front of the transcription start as well as the codons for the first 40 amino acids of TrpE, was amplified. This 523-bp fragment was digested with Pst and EcoRI, and the resulting 418-bp fragment was cloned into pBluescript SKII$^+$.

Primer extension of the *trpE* transcript. Thirty picomoles of oligonucleotide labeled with 60 μCi of $[^{32}P]ATP$ and T4 polynucleotide kinase. The 200-μl labeled oligonucleotide fraction from a NICK column (Pharmacia, Uppsala, Sweden) contained between 1.5 × 10$^5$ and 3 × 10$^5$ cpm/μl. For RNA-DNA hybridization, 15 to 100 μg of total RNA was precipitated, resuspended in 30 μl of hybridization buffer (80% [vol/vol] deionized formaldehyde, 1 mM EDTA [pH 8.0], 0.4 M NaCl, 40 mM pipercaine-$N$-$N$-$N$'-$N$'-bis(2-ethanesulfonic acid) (PIPES) [pH 6.4]), mixed with 2 × 10$^3$ to 3 × 10$^5$ cpm of labeled oligomer, denatured for 2 min at 85°C, and incubated overnight at 50°C. After precipitation and washing, the pellet was resuspended in 100 μl of elution buffer (20 μl of RNase inhibitor, 20 μl of 5× first strand buffer [Gibco BRL, Paisley, England], 10 mM dithiothreitol, 0.5 mM each deoxynucleotide [dATP, dCTP, dGTP, and dTTP], 200 U of SuperScript Reverse Transcriptase II [Gibco BRL]). Primer extension products were analyzed on 6% (wt/vol) acrylamide sequencing gels. The blot was exposed for 1 to 3 days on XAR-5 film (Kodak, Rochester, N.Y.) for autoradiography. For quantitation of the signals, a Molecular Dynamics PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) was used.

**Cell extract preparation and enzyme assays.** The preparation of cell extract and the conditions used for assaying anthranilate synthase (EC 4.1.3.27) and tryptophan synthase (EC 4.2.1.20) have been described previously (11).

The tryptophanase assay used for the determination of the internal tryptophan pools in *M. thermoautotrophicum* cells was carried out at 37°C with E. coli tryptophanase (EC 4.99.9.1). In this assay (9) trypophan is degraded to indole, which is quantitated as described for the trypophan synthase assay. One unit of enzyme activity represents the transformation of 1 nmol of substrate per min under standard assay conditions.

Tryptophan transport assay. Transport experiments were carried out at 60°C under anaerobic conditions. Cells of a 150-ml culture of mid-log phase *M. thermoautotrophicum* were harvested, washed twice with unsupplemented minimal medium, and resuspended to reach an optical density at 600 nm (OD$_{600}$) of 4.0. Five milliliters of this cell suspension was injected in a sterile, anaerobic, 50-ml reaction vessel containing 150 to 300 ml of minimal medium (30) supplemented with 8 mM Na$_2$WO$_4$ and 5.6 mM Na$_2$SeO$_3$ (Fluka, Buchs, Switzerland). The flasks were gassed with 2 bars of H$_2$–CO$_2$ (80%:20% [vol/vol]). For growth of the tryptophan-auxotrophic mutant MBT2, the medium was supplemented with 5 mM L-Trp (Fluka). 5-Methyltryptophan-resistant mutants were routinely cultivated in minimal medium supplemented with 7 mM 5-methyl-DL-tryptophan.

**RESULTS**

**MICs of tryptophan analogs.** The MICs of 5-methyl-DL-tryptophan and 6-fluoro-DL-tryptophan were determined for wild-type *M. thermoautotrophicum* (MBT1) as the amount of analog leading to half-maximal OD$_{595}$ after 18 h of growth of the cells in supplemented minimal medium (0 to 1 mM analog). For 5-methyltryptophan the MIC was 700 μM, and for 6-fluorotryptophan it was 2.2 mM. Following N-methyl-N-nitro-N-nitrosoguanidine mutagenesis and selection for 5-methyl-tryptophan resistance, 13 5-methyltryptophan-resistant mutants were isolated on plates supplemented with 10× the MIC of the 5-methyltryptophan analog (7 mM), whereas no colonies of the wild-type organism could be detected on 5× or 10× MIC plates in control experiments. Three of these thirteen mutants (named MWR1, MWR4, and MWR8) were chosen for further characterization. Growth rates of the wild type and of the three mutants on supplemented and unsupplemented media are listed in Table 1. All mutants showed a slightly shorter doubling time (by 5 to 15%) in unsupplemented medium when compared to generation times determined in medium supplemented with 10× the MIC of 5-methyltryptophan. Furthermore, all MWR strains grew essentially uninhibited on 22 mM 6-fluorotryptophan (10× the MIC) (data not shown), indicating that the mutation(s) leading to 5-methyltryptophan resistance also leads to cross-resistance towards 6-fluorotryptophan.

Characterization of a tryptophan-specific transport system. Resistance to 5-methyltryptophan could result from a mutation preventing entry of 5-methyltryptophan into cells. Since no transport system for 1-tryptophan in methanogens has been characterized, we first checked whether wild-type cells of *M. thermoautotrophicum* are able to take up 1-tryptophan from their growth medium. Using $^{14}$C-labeled 1-tryptophan, we discovered an anaerobic, 1-tryptophan-specific, energy-dependent uptake system in *M. thermoautotrophicum*. Uptake obeyed Michaelis-Menten kinetics, with a $K_m$ of 430 μM and a $V_{max}$ of 7.1 nmol of L-tryptophan/(mg [dry weight]/min) (Fig. 1). The specificity of this novel tryptophan uptake system was tested by providing L-phenylalanine, L-tyrosine, L-arginine, L-leucine, and D-tryptophan in molar excess over the substrate and measuring their effects on the 1-tryptophan transport rate. Of the amino acids tested, only L-tyrosine at a 3-fold molar excess and L-arginine at a 40-fold molar excess slightly competed with 1-tryptophan for transport. At a 40-fold molar excess, the d-enantiomer of tryptophan did not compete with 1-tryptophan for uptake, indicating that the tryptophan transport system of *M. thermoautotrophicum* is stereospecific.

Removal of H$_2$ from the headspace gas, exposure to air, or inhibition of methanogenesis by bromoethanesulfonic acid, a

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>Doubling time (h)$^a$</th>
<th>MMM</th>
<th>MMM + 5-MT</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBT1</td>
<td>Wild-type</td>
<td>4.1</td>
<td>58$^b$</td>
<td>58$^b$</td>
</tr>
<tr>
<td>MWR1</td>
<td>5-MT$^c$</td>
<td>3.9</td>
<td>4.1</td>
<td>4.1</td>
</tr>
<tr>
<td>MWR4</td>
<td>5-MT$^c$</td>
<td>4.1</td>
<td>4.8</td>
<td>4.8</td>
</tr>
<tr>
<td>MWR8</td>
<td>5-MT$^c$</td>
<td>3.7</td>
<td>4.3</td>
<td>4.3</td>
</tr>
</tbody>
</table>

$^a$ + 5-MT, addition of 7 mM 5-methyltryptophan (10× the MIC) of MMM, minimal medium.

$^b$ Extrapolation of the initial growth rate.

$^c$ 5-MT, 5-methyltryptophan-resistant.
shown to be transported by the same uptake system as L-tryptophan. The uptake activity was measured at a concentration of L-tryptophan (100 μM, dotted line). Error bars show the 90% confidence interval of the data.

**TABLE 2. Tryptophan uptake rates in wild-type and 5-methyltryptophan-resistant mutants of M. thermoautotrophicum**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Uptake rate (%) at 100 μM L-tryptophan</th>
<th>Uptake rate (%) at 750 μM L-tryptophan</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBT1</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>MBT2</td>
<td>ND</td>
<td>105</td>
</tr>
<tr>
<td>MWR1</td>
<td>64</td>
<td>113</td>
</tr>
<tr>
<td>MWR4</td>
<td>66</td>
<td>110</td>
</tr>
<tr>
<td>MWR8</td>
<td>53</td>
<td>86</td>
</tr>
</tbody>
</table>

The data shown are means from at least two independent samples of the same assay.

**FIG. 1. Rates of L-[14C]tryptophan uptake in wild-type M. thermoautotrophicum as a function of L-Trp concentration.**

**FIG. 2. Time curves for L-[14C]tryptophan uptake in wild-type M. thermoautotrophicum cells.**

Thus, the $K_i$ of 5-methyl-l-tryptophan is probably only about 30-fold higher than the $K_i$ for l-tryptophan (9 mM).

**Determination of l-tryptophan transport in 5-methyltryptophan-resistant mutants.** The rates of l-tryptophan transport in wild-type M. thermoautotrophicum (MBT1), in a tryptophan-auxotrophic mutant (MBT2), and in the three 5-methyltryptophan-resistant mutants, MWR1, MWR4, and MWR8, were determined at two different substrate concentrations, 100 and 750 μM l-tryptophan (Table 2). At the lower L-tryptophan concentration, the uptake rates in all MWR mutants were reduced by 40 to 50%, whereas transport rates at the higher L-tryptophan concentration were comparable to that of the wild-type for all mutants. Resistance to 5-methyltryptophan in the mutants is therefore unlikely to stem from a defect in l-tryptophan transport, as the three mutants were isolated independently from one another. The lower uptake rate in the MWR mutants at a low substrate concentration may be due to the higher internal tryptophan pools (see Table 4) as compared to the wild type.

**High, constitutive tryptophan enzyme activities in 5-methyltryptophan-resistant mutants.** Enzyme activities of anthranilate synthase and tryptophan synthase were measured in the wild type and 5-methyltryptophan-resistant mutants of M. thermoautotrophicum. In the wild type (MBT1) and the tryptophan-auxotrophic mutant (MBT2), supplementation of the growth medium with 5 mM l-tryptophan resulted in reduced activity of the anthranilate synthase and tryptophan synthase enzymes (Table 3). All MWR mutants showed constitutive activities of both enzymes, which were unaffected by the supplementation of the growth medium with 5 mM l-tryptophan or 7 mM 5-methyltryptophan.

In the MWR mutants, both enzymes were expressed at intermediate levels when compared to specific activities in tryptophan-repressed and tryptophan-starved MBT2 cells, although different levels were observed in different MWR mutants. In MWR4, l-tryptophan was found to slightly enhance expression of both anthranilate synthase and tryptophan synthase, an observation that remains unaccounted for. MWR1, which displays the most clear-cut constitutive phenotype, was used for further investigations.

**Internal tryptophan pools.** Relatively high constitutive activities of anthranilate synthase and tryptophan synthase measured in the MWR mutants can be expected to provide cells with larger intracellular l-tryptophan pools than in the wild type, which is reflected in the higher internal tryptophan pools (see Table 4) as compared to the wild type.
TABLE 3. Activity levels of anthranilate synthase and tryptophan synthase in wild-type M. thermoautotrophicum and 5-methyltryptophan-resistant mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Supplement during incubation</th>
<th>Relative sp act (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AS</td>
</tr>
<tr>
<td>MBT1</td>
<td>None</td>
<td>10.5 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>5 mM L-Trp</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>MBT2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>None</td>
<td>100 ± 15</td>
</tr>
<tr>
<td></td>
<td>5 mM L-Trp</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>MWR1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>None</td>
<td>18.9 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>5 mM L-Trp</td>
<td>20.2 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>7 mM 5-MT</td>
<td>19.1 ± 3.0</td>
</tr>
<tr>
<td>MWR4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>None</td>
<td>4.1 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>5 mM L-Trp</td>
<td>7.9 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>7 mM 5-MT</td>
<td>4.8 ± 1.1</td>
</tr>
<tr>
<td>MWR8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>None</td>
<td>11.3 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>5 mM L-Trp</td>
<td>12.7 ± 4.3</td>
</tr>
<tr>
<td></td>
<td>7 mM 5-MT</td>
<td>11.2 ± 0.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> A relative specific activity of 100 corresponds to 12.6 U/mg of protein for anthranilate synthase (AS) (MBT2, tryptophan-starved) and 48 U/mg of protein for tryptophan synthetase (TS) (MBT2, tryptophan starved). Assays were carried out aerobically at 37°C. The data shown are means ± standard deviations from two independently grown cultures.

<sup>b</sup> Cultivation was carried out in minimal medium supplemented with 5 mM L-Trp. At an OD<sub>600</sub> of approximately 1.0, cells were anaerobically harvested, washed twice with 1 volume of ununsupplemented minimal medium, and suspended in the incubation medium. After 15 h of incubation cells were harvested and used for the preparation of cell extracts.

<sup>c</sup> All the MWR mutant strains were cultivated on unsupplemented minimal medium, harvested at an OD<sub>600</sub> of approximately 1.0, and transferred into the indicated incubation medium. After 15 h of incubation cells were harvested and used for the preparation of cell extracts.

The aim of the present study was to investigate the regulatory mechanisms of tryptophan synthesis in M. thermoautotrophicum. Mutants resistant to 5-methyltryptophan were isolated following chemical mutagenesis with N-methyl-N′-nitro-N-nitosoguanidine and characterized in terms of L-tryptophan transport, enzyme activities, and transcription levels.

The MIC for 5-methyltryptophan in M. thermoautotrophicum (700 μM) is very similar to the MIC of 900 μM found in Methanococcus voltae (12). Optimal growth of the tryptophan-auxotrophic mutant, MBT2, was reported to require 5 to 10 mM L-Trp in the medium (20). This requirement for high concentrations of L-tryptophan initially suggested that the compound passively diffuses in and out of the cell. However, in this work, M. thermoautotrophicum Marburg was found to possess a transport system for tryptophan with a K<sub>m</sub> of 300 μM and a V<sub>max</sub> of 7 nmol/mg (dry weight)/min. We have not investigated whether or not the tryptophan transport system is sodium dependent. However, its energy requirement as well as its high K<sub>m</sub> are consistent with characteristics of secondary tryptophan transport systems described for the Archaea Halobacterium halobium (24) and Halobacterium salinarum (23) and of other archaeal secondary amino acid transport systems which have been shown to depend on sodium as a coupling ion (8, 17, 32). Transport was specific for the L form of tryptophan, and only L-tyrosine and L-arginine weakly competed with L-tryptophan for uptake. Stereospecificity of an uptake system is not unusual in prokaryotes and has been described for Pseudomonas acidovorans for tryptophan transport (28) as well as for Corynebacterium glutamicum for the transport of branched-chain amino acids (7). Different uptake systems for a given compound can be detected in a linearized

**TABLE 4. Internal L-tryptophan pools of M. thermoautotrophicum**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Internal L-Trp concentration (μM)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBT1</td>
<td>11.3 (9.9, 12.6)</td>
</tr>
<tr>
<td>MBT2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.8 (3.1, 4.4)</td>
</tr>
<tr>
<td>MWR1</td>
<td>45.3 (31.8, 58.8)</td>
</tr>
<tr>
<td>MWR4</td>
<td>17.1 (12.1, 22.0)</td>
</tr>
<tr>
<td>MWR8</td>
<td>24.3 (17.7, 37.2)</td>
</tr>
</tbody>
</table>

<sup>a</sup> L-Trp pools were estimated using an internal cell volume of 73.5 μL/mg (dry weight) (33) for cells grown without L-Trp supplementation, and the L-Trp content in crude extracts of M. thermoautotrophicum cultures was quantified with E. coli tryptophanase. Data are means of two independent measurements of the crude extract; the values of these measurements are shown in parentheses.

<sup>b</sup> Cells of the tryptophan-auxotrophic mutant MBT2 were grown on minimal medium supplemented with 5 mM L-Trp, starved for 15 h in unsupplemented medium, and used for the preparation of extracts.
plot of the Michaelis-Menten curve if their kinetic parameters are different (5). In the case of *M. thermoautotrophicum*, however, there was no kinetic evidence for the existence of more than one L-tryptophan uptake system.

Measurements of anthranilate synthase and tryptophan synthase specific activities in MWR mutants indicated constitutive activity of both enzymes, independently of the supplementation of the growth medium with L-Trp. It was not investigated whether 5-methyltryptophan resistance also abolished the previously detected effect of leucine starvation on the specific activities of these enzymes (11). The loss of tryptophan-regulated formation of anthranilate synthase and tryptophan synthase is consistent with mutations in a cis-acting regulatory feature of the trpE upstream region, in a regulatory protein, or in tryptophanyl-tRNA synthetase. An attempt was made to discriminate between these possibilities by sequence analysis of the trpE promoter regions of the mutant strains. In two of the three 5-methyltryptophan-resistant mutants different single-base-pair mutations were detected in this region. This suggested that constitutive expression of the tryptophan biosynthetic enzymes in these mutants was caused by alterations in the trpE promoter region. However, since all mutants were obtained after mutagenesis with N-methyl-N’-nitro-N-nitrosoguanidine, the possibility exists that either of these mutations (or both) are unrelated to the 5-methyltryptophan-resistant phenotype selected for. In the absence of a system for gene cloning in *M. thermoautotrophicum*, the specificity of the detected mutations on the level of tryptophan gene expression would have to be determined in an in vitro transcription system.

**ACKNOWLEDGMENT**

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**REFERENCES**


**FIG. 3.** Partial sequences of the putative trp operator regions of wild-type *M. thermoautotrophicum* and of two 5-methyltryptophan-resistant mutants. Nucleotides are numbered (5’→3’) with respect to the transcription start site (arrow). Archaeal box A and box B are boxed. Asterisks above the sequences indicate positions at which a mutation was found. Both strands were sequenced for each clone from two independent PCR.

**FIG. 4.** Primer extension of trpE mRNA in wild-type (MBT1) cells and tryptophan-auxotrophic (MBT2) and 5-methyltryptophan-resistant (MWR1) mutants of *M. thermoautotrophicum*. The primer extension product is shown with an arrow. The nucleotide sequence around the transcriptional start site is indicated in bold letters, and an asterisk shows the G nucleotide at the transcription start point. Lane 1, MBT1 incubated in minimal medium; lane 2, MBT1 in minimal medium + 5 mM L-Trp; lane 3, MBT2 in minimal medium; lane 4, MBT2 in minimal medium + 5 mM L-Trp; lane 5, MWR1 in minimal medium; lane 6, MWR1 in minimal medium + 5 mM L-Trp.


