A Gene Encoding L-Methionine γ-Lyase Is Present in
Enterobacteriaceae Family Genomes: Identification
and Characterization of Citrobacter freundii
L-Methionine γ-Lyase
Ilya V. Manukhov,1 Daria V. Mamaeva,2 Sergei M. Rastorguev,1 Nicolai G. Faleev,3 Elena A. Morozova,2 Tatyana V. Demidkina,2† and Gennadii B. Zavilgelsky1*†
State Research Institute of Genetic and Selection of Industrial Microorganisms, 1st Dorozhnii pr. 1, Moscow 117545, Russia,1 Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Vavilov str., 32, Moscow 119991, Russia,2 and Nesmeyanov Institute of Elementoorganic Compounds, Russian Academy of Sciences, Vavilov str., 28, Moscow 119991, Russia3

Received 11 November 2004/Accepted 21 February 2005

Citrobacter freundii cells produce L-methionine γ-lyase when grown on a medium containing L-methionine. The nucleotide sequence of the hybrid plasmid with a C. freundii EcoRI insert of about 3.0 kbp contained two open reading frames, consisting of 1,194 nucleotides and 1,296 nucleotides, respectively. The first one (denoted megL) encoded L-methionine γ-lyase. The enzyme was overexpressed in Escherichia coli and purified. The second frame encoded a protein belonging to the family of permeases. Regions of high sequence identity with the 3′-terminal part of the C. freundii megL gene located in the same regions of Salmonella enterica serovar Typhimurium, Shigella flexneri, E. coli, and Citrobacter rodentium genomes were found.

L-Methionine γ-lyase (MGL; EC 4.4.1.11) is a pyridoxal 5′ phosphate (pyridoxal-P)-dependent enzyme catalyzing the γ elimination of L-methionine to produce methanethiol, ammonia, and α-ketobutyrate (Fig. 1).

The enzyme also catalyzes the β elimination reaction of L-cysteine and S-substituted L-cysteines as well as the γ and β replacement reactions of L-methionine and L-cysteine and their analogues (11).

MGL has been isolated from Pseudomonas putida (8), from some other bacteria, and from the primitive protozoa Entamoeba histolytica (12) and Trichomonas vaginalis (6).

Citrobacter intermedius cells produce MGL when grown on a medium containing lactate and L-methionine (3). This finding was the only indication of the presence of MGL in the Enterobacteriaceae family. In the present work, we have purified MGL from Citrobacter freundii cells and have cloned and sequenced the 3.0-kbp insert which contained two open reading frames (ORFs), the first one encoding MGL and the second one encoding a putative permease. For overproduction of the C. freundii MGL, the plasmid pET-mgl has been constructed and homogeneous recombinant MGL (rMGL) has been obtained. Kinetic parameters for γ and β elimination reactions catalyzed by both wild-type enzyme and rMGL have been determined.

In the genomes of Escherichia coli, Shigella flexneri, Salmonella enterica serovar Typhimurium, and Citrobacter rodentium, we found the regions with high sequence identity to the 3′-terminal region of the megL gene located upstream of the gene encoding a putative permease, as that seen in C. freundii.

**Purification of MGL from C. freundii.** C. freundii cells (ATCC 29063) were grown in the presence of 0.4% DL-methionine as described in reference 3. The elaborated procedure for purification of MGL is presented in Table 1. All operations were performed at 4°C. One unit of activity was defined as the amount of enzyme which catalyzes the formation of 1.0 μmol of α-ketobutyrate at 30°C per minute. The specific activity was expressed as units per mg of protein. Determination of α-ketobutyrate was performed with 2,4-dinitrophenylhydrazine (4). According to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the enzyme’s preparations were 86% pure and the molecular mass of a subunit was estimated to be 43 to 45 kDa.

To determine the N-terminal sequence of the enzyme, it was additionally purified by nondenatured polyacrylamide gel electrophoresis. The enzyme was electroblotted from the gel onto

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Sp act (U/mg of protein)</th>
<th>Activity yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>414</td>
<td>189</td>
<td>0.46</td>
<td>100</td>
</tr>
<tr>
<td>Precipitation</td>
<td>409</td>
<td>188</td>
<td>0.46</td>
<td>99</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>129</td>
<td>184</td>
<td>1.43</td>
<td>97</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>27</td>
<td>122</td>
<td>4.5</td>
<td>65</td>
</tr>
<tr>
<td>Sephacryl S-200 HR</td>
<td>4</td>
<td>40</td>
<td>10.0</td>
<td>21</td>
</tr>
</tbody>
</table>

† T.V.D. and G.B.Z. contributed equally to this paper.

* Corresponding author. Mailing address: State Research Institute of Genetic and Selection of Industrial Microorganisms, 1st Dorozhnii pr. 1, Moscow 117545, Russia. Phone: (7095) 315 37 29. Fax: (7095) 315 05 01. E-mail: zavigel@genetika.ru.

Copyright © 2005, American Society for Microbiology. All Rights Reserved.
a polyvinylidene fluoride membrane according to the method described in reference 9, and the sequence of amino acid residues obtained by automatic sequencing was MSDCRTYG.

Cloning of the C. freundii gene encoding MGL. To screen the C. freundii genomic DNA for the gene encoding MGL, we used degenerate primers Nvir and Avir for PCR amplification of approximately 470 bp of the 5′/H11032 tail fragment of the megL gene. Primer Nvir (5′/H11032-ACITAYAARTTYAARTTYAAYACICARATHGT-3[H11032][26 bp], where Y is C or T; R is A or G; H is A, C, or T; and I is A, G, C, or T) corresponds to the N-terminal amino acid sequence of MGL. Primer Avir (5′/H11032-GGRTTIGCIGGIGTYTCRTTRTA-3[H11032]) corresponds to the conservative amino acid sequence YFETPANP of P. putida MGL (5). Following separation of the DNA fragments in an agarose gel, a band of DNA of about 500 bp was isolated, ligated into pUC18 with blunt ends, and introduced into E. coli TG1 cells. E. coli cells were grown on Luria-Bertani medium in the presence of ampicillin. Several clones containing the plasmids with DNA fragments of about 500 bp were obtained. DNA sequencing showed that one of these fragments is homologous to the 5′/H11032 end of the P. putida gene encoding MGL (5). Using this sequence, we prepared the primers Ndir (5′/H11032-GAGCCTTCTACAGGCGCGGGTAG-3[H11032]) and Nrev (5′/H11032-GATATACACTACTTTGGTTTCCGG-3[H11032]). In order to clone the entire megL gene, we constructed a library in the pUC18 vector containing EcoRI fragments of C. freundii chromosomal DNA. Colonies from petri dishes containing about 1,000 clones per petri dish were replica plated. The primary dish was split into sectors of approximately 100 colonies each, which were suspended in water, and 5 µl of this suspension was used as template for PCR amplification. The amplification was conducted using two primers, Ndir and Nrev. Agarose gel electrophoresis revealed that colonies from two sectors gave a PCR product of about 500 bp. The same procedure (PCR amplification with Ndir and Nrev primers followed by identification of 500-bp fragments in an agarose gel) was applied to each of the 100 colonies from the two selected sectors. Finally, two clones which carried plasmids with the same EcoRI fragment in pUC18 (of about 3.0 kbp) were selected.

Nucleotide sequence of the C. freundii 3.0-kbp fragment. The nucleotide sequence of the 3.0 kbp fragment was determined on both strands. It contained two ORFs: ORF1 of 1,194 nucleotides, starting with the ATG initiation codon and ending with the TGA terminator codon, at position 1445, and ORF2, starting with an ATG initiation codon, located at position 1563 of the 3′ region of the ORF1. The region of 1,194 nucleotides encoded a protein of 398 amino acid residues. This protein was identified as MGL based on a computer search of GenBank. The gene encoding the enzyme was designated megL. The sequence of the ORF2 encoded a protein of 432 amino acid residues. A computer search of GenBank found that this protein belongs to a family of permeases. This gene was designated aap. The nucleotide sequences of the upstream region and the spacer between the megL and aap genes of C. freundii are shown in Fig. 2. In the sequence preceding the megL gene, the putative elements of the regulatory region are shown, namely the ribosome binding site and −10 and −35 sites of a putative promoter. Downstream of the megL gene is an inverted repeat sequence of 28 bp, and an initiation ATG codon.

CTGCACCCTA GTTCTACGGT AGCACCCTTG CCATACACT GCCCAATACA CGGCTCTGCC
TAGGCGCTG CATTCCACCT GTGTTCTCAC CATTACGTGG AGATTTCTTG CCGATTGGCA
ACATTCGTC CGGTCTCTCG AGTACAGCT GCCAGTTGGC AACATCTCIG CGGCTCTTC
−35
−10
GATGACAGAA AAGACATAT CTGGATTGT ATCTATATTG GATTTCTGTT GCACAGCGAT

rbs
TGGCCTGATT CCGCCCTCGA ACTATCTGGT AGGAATCGA GGGAGATTAC ATG megL TGA
CCATTATGAC CTCGAACCCAC GCCGAACGGT TCAGTTGTT GAAATCAAG GCACACTG
CTGATTAATAC GCCCAATCCT GTTCGGGGA GAGCGCGTATTTTGGCAGGG GGTCT ATG aap TAA

FIG. 1. MGL, a pyridoxal-P-dependent enzyme catalyzing the γ elimination of L-methionine to produce methanethiol, ammonia, and α-ketobutyrate.

FIG. 2. The nucleotide sequences of the upstream region and the spacer between the megL and aap genes of C. freundii. Genes megL and aap are shown as boxes with initiation and termination codons. The putative ribosome binding site (rbs) and promoter sites (−10 and −35) are underlined. The inverted repeat sequence (28 bp) between megL and aap is marked with arrows.
for ORF2 is located at 82 bp from the inverted repeat element. The sequence identity between MGLs from different species did not exceed 60%. Phylogenetic analysis of MGLs showed that there is no correlation between the amino acid sequence identity and the taxonomic position of species on the phylogenetic tree. One may assume that in these species genes coding MGL arose for the most part by horizontal transfer.

Production, purification, and kinetic parameters of MGL.

The DNA fragment containing the megL gene was amplified by PCR using primer Nspec (5′-GATATCATGGGCTGACTGT CGTACTTAC-3′), complementary to the 5′ terminus of the gene encoding MGL and containing the restriction site NcoI (underlined; the substituted nucleotide is shown in italics) and the reverse primer of pUC18. After isolation from agarose gel, the fragment was inserted into pET-15b via NcoI and BamHI sites. Following transformation of E. coli BL21 (DE3) cells, a clone containing the plasmid (named pET-mgL) was obtained. It produced MGL up to 20% of total cell protein. The purification procedure for rMGL was the same as for the wild-type enzyme except the heat treatment step was omitted. The enzyme with specific activity of 10 U/mg was obtained with a total yield of 36%. Steady-state kinetic parameters of the wild-type enzyme with specific activity of 10 U/mg was obtained with a total enzyme except the heat treatment step was omitted. The enzymatic procedure for rMGL was the same as for the wild-type clone containing the plasmid (named pET-mgL) was obtained.

The sequence identity between MGLs from different species may be seen that in the genomes of serovar Typhimurium, S. enterica, C. freundii, S. flexneri, and E. coli, the sequences homologous (especially in the serovar Typhimurium genome) to the sequences is observed. However, the sequence similarity is observed only for megL gene, which corresponds to 79 C-terminal amino acids of the MGL. As far as about 500 bp upstream of this site, any sequence similarity with the C. freundii megL gene is absent, although about 80% sequence similarity among serovar Typhimurium, S. flexneri, and E. coli sequences is observed.

To our knowledge, no data indicating the presence of the gene coding MGL in genomes of Enterobacteriaceae family were known. The results presented here allow a revision of the notion of the absence of a gene coding MGL in genomes of the Enterobacteriaceae. It is noteworthy that the megL gene is located in the same environment in the genomes of C. freundii, serovar Typhimurium, S. flexneri, E. coli, and C. rodentium, and the breaking of the megL gene and deletion of its 5′-terminal sequence in genomes of E. coli, S. flexneri, S. enterica serovar Typhimurium, and C. rodentium took place at the same site (Fig. 3).

We may suppose that in the process of evolution, the following events have taken place (Fig. 4). First, the megL gene was inserted into the genome of an ancestor 1 as a result of the horizontal transfer (stage I). Next, a certain branch (C. freundii) continued to exist with the complete and active megL gene. At stage II, the megL gene was broken (its 5′ fragment was deleted) in the genome of an ancestor 2 of E. coli, S. flexneri, and serovar Typhimurium. At stage III, the 5′ fragment was deleted in the genome of an ancestor 2 of E. coli, S. flexneri, and serovar Typhimurium. At the stage III, a deletion of the 3′ terminus of megL and the spacer in E. coli and S. flexneri genomes had taken place. It seems likely that a deletion of the megL gene and the spacer between the aap and megL genes in the C. rodentium genome is associated with a repeated horizontal transfer of this DNA fragment from the E. coli (or S. flexneri) genome. Probably, a definite role in these reconstructions was played by the inverted repeat element (28 bp) located immediately after the megL. It is known that inverted repeat sequences may determine the recombination’s “hot spots” (2).

The comparative analysis of nucleotide sequences of completely sequenced genomes of Erwinia carotovora, Klebsiella pneumoniae, Yersinia pestis, and Photobacterium luminescens has revealed the absence of sequences highly similar to the sequences of aap and megL. This may be considered as evidence that the horizontal transfer and insertion of this cluster into the genome of ancestor 1 (Fig. 4) had taken place after the divergence of these species in the course of evolution. We may only speculate as to the reasons that have led to the destruction of the megL gene in E. coli, S. flexneri, serovar Typhimurium, and C. rodentium and ensured its stable existence in the genome of C. freundii. This may be associated with the influence of the megL gene on the intracellular content of S-adenosylmethionine (AdoMet). Being a donor of the methyl group, AdoMet belongs to a group of molecules participating in a number of important processes in cells, and any decrease in the levels of AdoMet should lead to a loss of the cell’s viability (10). The reason for its retention in the genome of C. freundii may be

### Table 2. Kinetic parameters of β and γ elimination reactions for C. freundii MGLs

<table>
<thead>
<tr>
<th>Substrate</th>
<th>C. freundii, wild-type MGL</th>
<th>C. freundii, rMGL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{cat}$ (s$^{-1}$)</td>
<td>$K_m$ (mM)</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>6.5 ± 0.12</td>
<td>0.7 ± 0.05</td>
</tr>
<tr>
<td>Ot-Homocystine</td>
<td>5.1 ± 0.16</td>
<td>1.1 ± 0.13</td>
</tr>
<tr>
<td>L-Ethionine</td>
<td>6.2 ± 0.28</td>
<td>0.56 ± 0.09</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>2.24 ± 0.13</td>
<td>0.18 ± 0.02</td>
</tr>
<tr>
<td>S-Me-L-cysteine</td>
<td>5.97 ± 0.08</td>
<td>0.61 ± 0.02</td>
</tr>
<tr>
<td>S-Et-L-cysteine</td>
<td>6.8 ± 0.19</td>
<td>0.49 ± 0.02</td>
</tr>
<tr>
<td>S-Bzl-L-cysteine</td>
<td>10.9 ± 0.71</td>
<td>0.19 ± 0.01</td>
</tr>
</tbody>
</table>

* Kinetic parameters of β elimination reactions have been determined by 2,4-dinitrophenylhydrazine; kinetic parameters of γ elimination reactions have been determined by coupled assay with L-lactate dehydrogenase and NADPH (7).
FIG. 3. Alignment of the *C. freundii*, serovar *Typhimurium*, *S. flexneri*, *E. coli*, and *C. rodentium* 3′-terminal fragments of the *megL* gene, spacer, and 5′ fragment of *aap*. Nucleotide substitutions encountered in the DNA of serovar *Typhimurium* (S.t.), *S. flexneri* (S.f.), *E. coli* (E.c.), and *C. rodentium* (C.r.) are shown in lowercase letters. The spacer inverted repeat sequence (28 bp) is italicized. The TGA stop codon of *megL* and the ATG initiation codon of *aap* are italicized and underlined. GenBank nucleotide and genome accession number information is as follows: for *C. freundii*, AY204910; for serovar *Typhimurium*, NC_003197; for *E. coli* K12, NC_000913; for *S. flexneri*, NC_004337; for *C. rodentium*, http://www.sanger.ac.uk/Projects/C_rodentium; for *E. carotovora* subsp. *atroseptica* SCRI1043, NC_004547; for *P. luminescens*, NC_000913; for *K. pneumoniae* subsp. *pneumoniae* MGH 78578, NC_002941; and for *Y. pestis* KIM, NC_004088.
particularities of regulation of gene’s expression, namely the existence of a repression system to the effect that the megL gene opens only in the presence of a comparatively large concentration of L-methionine, and, consequently, its activity cannot decrease the intracellular pool of AdoMet to a critical value.

**Nucleotide sequence accession number.** The nucleotide sequences reported in this paper have been submitted to GenBank with accession number AY204910.

This work was supported in part by grants from Russian Foundation for Basic Investigations (05-04-48010 for T.V.D. and 03-04-48189 for G.B.Z.), the State Program for Support of Leading Scientific Schools (1800.2003.4 for T.V.D. and D.V.M.; 978.2003.4 for G.B.Z., I.V.M., and S.M.R.), and the Fogarty International Foundation (TW00106 and TW006045 for T.V.D).

We thank R.S. Phillips for the kind gift of the *C. freundii* strain. We are grateful to Paul D. Gollnick for critical reading of the manuscript.

**REFERENCES**