Cloning and Sequencing of *Escherichia coli* murZ and Purification of Its Product, a UDP-N-Acetylglucosamine Enolpyruvyl Transferase

JOHN L. MARQUARDT, DEBORAH A. SIEGELE, ROBERTO KOLTER, AND CHRISTOPHER T. WALSH*  

Departments of Biological Chemistry and Molecular Pharmacology and Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

Received 16 April 1992/Accepted 1 July 1992

The *Escherichia coli* gene murZ, encoding the enzyme UDP-N-acetylglucosamine enolpyruvyl transferase, has been cloned and sequenced. Identified by screening an *E. coli* genomic library for clones that conferred phosphomycin resistance, murZ encoded a 419-amino-acid polypeptide and was mapped to 69.3 min on the *E. coli* chromosome. MurZ protein was purified to near homogeneity and found to have the expected UDP-N-acetylglucosamine enolpyruvyl transferase activity. Sequence analysis of the predicted product revealed 44% identity to OrfR from *Bacillus subtilis* (K. Trach, J. W. Chapman, P. Piggot, D. LeCoq, and J. A. Hoch, J. Bacteriol. 170:4194-4208, 1988), suggesting that orfR may also encode a UDP-N-acetylglucosamine enolpyruvyl transferase enzyme. MurZ is also homologous to the aromatic amino acid biosynthetic enzyme enolpyruvyl shikimate phosphate synthase, the other enzyme known to catalyze an enolpyruvyl transfer.

The chemical structure of the bacterial cell wall imparts rigidity to the cell, protects it against osmotic lysis, and determines the bacterial cell shape (14, 15). The major structural element of the cell wall is the polymeric peptidoglycan murein. In *Escherichia coli* it consists of alternating units of N-acetylglucosamine (GlcNAc) and N-acetylmuramid (MurNAc) with an attached pentapeptide. The enzyme UDP-GlcNAc enolpyruvyl transferase catalyzes the first committed step in peptidoglycan assembly and is the target for the antibiotic phosphomycin (8) (Fig. 1). Phosphomycin causes a time-dependent inactivation of the enzyme, presumably by binding to the active site as a phospho-enolpyruvylate (PEP) analog and undergoing covalent capture by the enzyme with epoxide ring opening (8). We exploited this fact in developing our experimental strategy to clone the *E. coli* gene encoding UDP-GlcNAc enolpyruvyl transferase. Since phosphomycin leads to inactivation by covalent modification of the enzyme, increased synthesis of the protein by cloning the gene into a multicopy plasmid could lead to a resistant phenotype.

A genomic library of *E. coli* AB1157 was made by using the restriction endonuclease PstI and the multicopy plasmid pUC19 (23). The library was screened for clones resistant to phosphomycin. Approximately 4,000 transformants were replica plated onto Luria-Bertani plates containing 120 μg of phosphomycin per ml, and 14 phosphomycin-resistant clones were isolated. Ten of the 14 phosphomycin-resistant (Fos') clones were also maltose negative (Mal') on MacConkey maltose plates, suggesting that they have defects in adenylate cyclase or the catabolite gene activator protein, which positively regulates the genes involved in transport of both phosphomycin and maltose (1, 8, 17). These were not studied further.

In order to confirm that the Fos' phenotype was due to the cloned chromosomal DNA, plasmid DNA from the four Fos' Mal' clones was isolated and used to retransform AB1157 (Table 1). In each case the transformants were Fos'.

The four transformants were then assayed for UDP-GlcNAc enolpyruvyl transferase activity. Three-milliliter overnight cultures were grown, harvested by centrifugation, washed, and resuspended in 1 ml of buffer A (50 mM Tris-HCl, pH 8.0, and 5 mM dithiothreitol). The resuspended cells were then sonicated by three 15-s pulses at full power with an MSE Ultrasonic microtip sonicator. Cell debris was pelletted by centrifugation, and the supernatant was removed. The supernatant was desalted on a Pharmacia NAP10 column equilibrated with buffer A.

Enzymatic activity was assessed by quantitating the UDP-GlcNAc-dependent release of P, from PEP. Seventy microliters of sample was added to 30 μl of an assay mix containing 10 μl of 250 mM Tris (pH 7.8), 10 μl of 100 mM UDP-GlcNAc, and 10 μl of 100 mM PEP. This mixture was incubated at 37°C, and 10-μl aliquots were removed at 0, 15, 30, and 45 min and assayed for P, by the method of Lanzetta et al. (9). Eight hundred microliters of Lanzetta's malachite green-ammonium molybdate assay mix was added to each 10-μl aliquot. P, was quantitated by measuring the optical A660. Sterox detergent and citric acid were omitted from the method of Lanzetta et al.

The specific activity of one clone which contained plasmid pJLMO01 was 20- to 40-fold higher than the specific activity of AB1157 transformed with pUC19 alone. The specific activity of the other three clones was comparable to that of AB1157 transformed with pUC19 alone.

Plasmid DNA was purified from these strains, and restriction maps were constructed. Plasmid pJLMO01 contains a 9.0-kb PstI fragment. Subclones of this PstI fragment were made and tested for the ability to confer phosphomycin resistance. The Fos' gene was localized to a 4.5-kb PstI-BamHI fragment cloned in the PstI and BamHI sites of pUC19. This plasmid was named pJLMO03. The inserts of each of the remaining three plasmids differed from one another and from pJLMO01. The mechanism of Fos' conferred by these three clones remains to be determined.

Insertion mutagenesis of pJLMO03 with the transposon Tn1000 was done to further localize the gene responsible for the Fos' phenotype. Tn1000 insertions into pJLMO03 were

* Corresponding author.
made by the method of Guyer (7), and the insertion mutants were tested for the ability to confer phosphomycin resistance. Twenty-two Tn1000 transposon insertions into pJLM003 resulted in a loss of phosphomycin resistance and were localized to a 1.4-kb segment by restriction mapping.

The 1.4-kb region was sequenced by the chain-terminating method with primers homologous to either the left or right end of Tn1000 (10). Eight Tn1000 insertions, spaced 200 to 250 bp apart, were used as priming sites. The overlapping sequence of both DNA strands was obtained for 1,396 bp. In addition, an approximately 200-base single-stranded sequence at each end was obtained (Fig. 2). Three hundred eighty-nine base pairs of this sequence overlapped with sequence downstream of the nlp gene (4). This sequence overlap localized the chromosomal insert in pJLM003 to 69.3 min on the E. coli genetic map.

The map position of murZ differs from the location of 90 min reported by Venkateswaran and Wu for murA, encoding a UDP-GlcNAc enolpyruvate transferase necessary for cell wall biosynthesis. They isolated and characterized a temperature-sensitive, phosphomycin-resistant murA mutant of E. coli and demonstrated that the UDP-GlcNAc enolpyruvate transferase from the mutant strain was resistant to phosphomycin and the substrate PEP (20, 22). The different map positions of the murA and murZ genes suggest that E. coli may have two UDP-GlcNAc enolpyruvate transferases. The physiological role of the murZ gene product remains to be determined.

The sequenced region contained a 1,257-bp open reading frame that could encode a 419-amino-acid protein with a predicted molecular mass of 44,800 Da (Fig. 2). This open reading frame was designated murZ. Three of the Tn1000 insertions in pJLM003 that caused a Fosβ phenotype are upstream of this coding region and presumably interfere with expression of murZ.

To test the hypothesis that murZ encodes a UDP-GlcNAc enolpyruvate transferase, the murZ gene product was purified and characterized biochemically. The murZ gene was cloned into the expression vector pKen under the control of the inducible lac promoter (11, 16). In the presence of the inducer isopropyl-β-D-thiogalactopyranoside (IPTG), XA90 cells containing this plasmid, pJLM004, overproduced a protein of approximately 44 kDa (Fig. 3, compare lanes 2 and 3). One liter of JLM 16, the overproducing strain, was grown in LB at 37°C to an optical density at 595 nm of 0.5, induced with 10 ml of 100 mM IPTG, and harvested after 6 h of growth. Cells were harvested by centrifugation, washed with cold 100 mM Tris-Cl (pH 8.0)-5 mM dithiothreitol, and then lysed by three passages through a French press. Cell debris was pelleted by centrifugation. One milligram of bovine pancreatic DNase I and 1 mg of bovine pancreatic RNase A were added to the supernatant and incubated for 1 h at 4°C. A 70% ammonium sulfate precipitation was resuspended in a minimal volume and desalted on a G-25 column. Protein was loaded onto a Pharmacia Hi-Load Q 10/25 column and purified to near homogeneity by using a gradient of KCl from 0 to 1 M (Fig. 3, lane 4). The amino-terminal sequence of the first 10 residues of the purified protein, determined by the Harvard Micro-Chemistry Facility, was identical to the sequence predicted from the DNA and identified the protein as the murZ product.

The purified MurZ protein had the expected UDP-GlcNAc enolpyruvate transferase activity. The kcat for the transfer of enolpyruvate from PEP to UDP-GlcNAc was 285 min⁻¹. The Km for UDP-GlcNAc was 2.5 mM, and the Km for PEP was 1.0 mM. As shown in Fig. 4, the purified enzyme was inactivated in a time-dependent manner by phosphomycin,

**TABLE 1. Bacterial strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB1157</td>
<td>F⁻ thr-1 ara-14 leuB6 Δ(gpt-pro)62 lacY1 tsx-33 supE44 galK2</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td></td>
<td>λ⁻ rac hisG4(Oc) rfdD1 mgl-51 rpsL31 hagK51 xyl-3 mit-1 argE3 thi-1</td>
<td></td>
</tr>
<tr>
<td>RDK1736</td>
<td>MG 1063 F⁺ recA56</td>
<td>6; obtained from R. Kolodner, Harvard Medical School</td>
</tr>
<tr>
<td>XA90</td>
<td>Δ(lac-pro) ara nalA argE(Am) thi rpoB (F' lacP3'Z⁺ Y⁺ proAB⁺)</td>
<td>G. Verdone, Harvard University</td>
</tr>
<tr>
<td>ZK4</td>
<td>MC4100 [F⁻ araD139 Δ(argF-lac)U169 rpsL150 relA1 ffb-5301 deoCl ptsF25 rbsR] recA56</td>
<td>R. Kolter</td>
</tr>
<tr>
<td>JLM2</td>
<td>RDK1736/pJLM003</td>
<td>This work</td>
</tr>
<tr>
<td>JLM16</td>
<td>XA90/pJLM004</td>
<td>This work</td>
</tr>
</tbody>
</table>

FIG. 1. Enzymatic reaction catalyzed by UDP-GlcNAc enolpyruvate transferase: product of the murZ gene.
and, like other bacterial UDP-GlcNAc enolpyruvate transferases, inactivation by phosphomycin was dependent on the concentration of UDP-GlcNAc (3, 20).

The translated murZ sequence was compared with sequences in the NBRF (PIR), SWISSPROT, and translated GenBank data banks by using the BLAST algorithm (2). Two sequences with high levels of amino acid sequence similarity to MurZ were reported. The protein with the highest sequence similarity to MurZ was the translated open reading frame of the Bacillus subtilis orfR gene, a gene of unknown function (19). OrfR and MurZ were 43.7% identical over 350 amino acids. This high degree of identity suggests that the orfR and murZ gene products will have similar activities.

The 5-enolpyruvyl shikimate-3-phosphate (EPSP) synthase of Bordetella pertussis (12) was also identified as similar to MurZ, with 16.1% identity between the two proteins over 442 amino acids. EPSP synthase is the only
other enzyme known to catalyze such a mechanistically unusual transfer of enolpyruvyl from PEP. EPSP synthase catalyzes the transfer of an enolpyruvyl group from PEP to

\[ \text{EPSP} \rightarrow \text{GluNAc} \]

the 5'-OH of shikimate-3-phosphate with concomitant release of P. EPSP is then a precursor to chorismate in the aromatic amino acid biosynthetic pathway. The E. coli EPSP synthase (AroA) (5) is 18.3% identical to MurZ over 420 amino acids.

Recently, a crystal structure of the E. coli EPSP synthase was reported at 3-Å (0.3-nm) resolution (18). The similarity of MurZ to the EPSP synthase family leads us to expect it to be structurally similar to the E. coli EPSP synthase. In the crystal structure of EPSP synthase a sixfold repeating structural unit is evident (18). The six units of roughly 70 amino acids are arranged into a two-domain structure, each domain formed from three units related by an approximate threefold symmetry axis. Stallings et al. (18) speculate that this may be the result of a gene duplication of a primordial 70-amino-acid folding unit.

The sequence similarity of MurZ to other EPSP synthases led us to look for a repeating unit in MurZ. By using the MACo algorithm to analyze the murZ amino acid sequence, six regions of internal homology were identified (13, 21). The 419-residue protein sequence was divided into six units of approximately 70 amino acids. Each unit contains a conserved motif, LXXLGPA-polar-hydrophobic-polar. A detailed structural analysis will be required to test the hypothesis that this predicted sixfold structural repeat is indeed present in the MurZ structure.

Nucleotide sequence accession number. The murZ sequence has been submitted to GenBank and has accession number M92358.

We acknowledge the useful insights and sequence analysis graciously provided by H. B. Nicholas, Jr., supported in part by NIH grant P41 RR06009 to the Pittsburgh Supercomputing Center. We thank Jim Hu for his critical reading of the manuscript.

This work was supported in part by NSF grants MBS8917290 (C.T.W.) and DMB8820458 (R.K.). D.A.S. was supported by NIH postdoctoral fellowship GM13781, and J.L.M. was supported by an NIH training grant and Ryan Fellowship administered by Harvard Medical School.

ADDENDUM IN PROOF

Wanke et al. (C. Wanke, R. Falchettor, and N. Amrhein, FEBS Lett. 301:271–276, 1992) have recently cloned, sequenced, and overexpressed a gene encoding a UDP-GlcNAc enolpyruvate transferase from Enterobacter cloacae. The encoded enzyme of E. cloacae is 94% identical to the E. coli MurZ.

REFERENCES


