

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

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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*-acetylmurein (MurNAc) with an attached pentapeptide. The enzyme UDP-GlcNAc enolpyruvate 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 phosphoenolpyruvate (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 enolpyruvate 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 *Pst*I 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*^r) 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*^r phenotype was due to the cloned chromosomal DNA, plasmid DNA from the four *Fos*^r *Mal*⁺ clones was isolated and used to retransform AB1157 (Table 1). In each case the transformants were *Fos*^r.

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 pelleted 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_i 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_i 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_i 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 pJLM001 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 pJLM001 contains a 9.0-kb *Pst*I fragment. Subclones of this *Pst*I fragment were made and tested for the ability to confer phosphomycin resistance. The *Fos*^r gene was localized to a 4.5-kb *Pst*I-*Bam*HI fragment cloned in the *Pst*I and *Bam*HI sites of pUC19. This plasmid was named pJLM003. The inserts of each of the remaining three plasmids differed from one another and from pJLM001. The mechanism of *Fos*^r conferred by these three clones remains to be determined.

Insertion mutagenesis of pJLM003 with the transposon Tn1000 was done to further localize the gene responsible for the *Fos*^r phenotype. Tn1000 insertions into pJLM003 were

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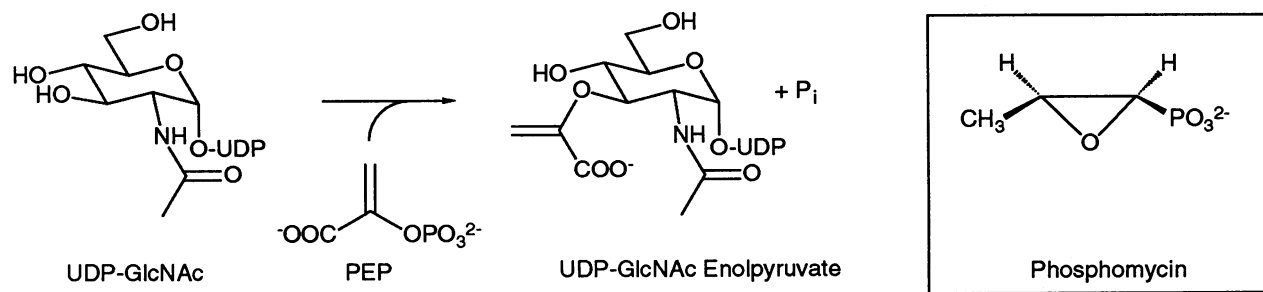


FIG. 1. Enzymatic reaction catalyzed by UDP-GlcNAc enolpyruvate transferase: product of the *murZ* gene.

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 in vitro and had reduced affinity for both 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 Fos^s 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 k_{cat} for the transfer of enolpyruvate from PEP to UDP-GlcNAc was 285 min⁻¹. The K_m for UDP-GlcNAc was 2.5 mM, and the K_m 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

| Strain | Description | Source or reference |
|---------|---|--|
| AB1157 | F ⁻ <i>thr-1 ara-14 leuB6 Δ(gpt-proA)62 lacY1 tsx-33 supE44 galK2 λ⁻ rac hisG4(Oc) rfbD1 mgl-51 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1</i> | American Type Culture Collection |
| RDK1736 | MG 1063 F ⁺ <i>recA56</i> | 6; obtained from R. Kolodner, Harvard Medical School |
| XA90 | <i>Δ(lac-pro) ara nalA argE(Am) thi rpoB (F' lacI^{ts}Z⁺Y⁺ proAB⁺)</i> | G. Verdine, Harvard University |
| ZK4 | MC4100 [F ⁻ <i>araD139 Δ(argF-lac)U169 rpsL150 relA1 ffb-5301 deoC1 ptsF25 rbsR</i>] <i>recA56</i> | R. Kolter |
| JLM2 | RDK1736/pJLM003 | This work |
| JLM16 | XA90/pJLM004 | This work |

1: CAGGGCAACAATGTGACGCTTCAGGGGTAACGACAAAGTGTATACCTGGCGAAATGTATAATTTGCCTGCTGATGTTCTGCCTCGTTAAATTTTTT

101: TCAGGCCGGATTATGCGTTAACCGCCTGTCCGGCAAACCTGCCTGCAAGTCGAGCCTTGTCTGATTTATCAGCGAGGCTTTTTGCTTATTATGCCAGC

201: GCAACTTTGCTCTAAGATGTTTCGCTGGTTATTAAGTATGATGATGAAGATCCCATGGAAAATAATGAAATTCAGAGCGTGTGATGAACGCTCTCTCCC

301: TCCAGGAAGTCCACGTTTCCGGCGATGGCAGCCACTTTTCAGGTTATGCCCCTGGGTGAGTTGTTTACGGCATGAGTCGGGTTAAAAACAGCAGACGGT

401: CTATGGTCCGCTGATGGAATATATTTGCGGATAACCCGATTCATGCTGTGTCGATCAAAGCGTATACCCCTGCGGAGTGGGCGCGCATCGCAAACCTGAAC

MurZ: m d k f r v q g p t k l q

501: GGCTTTGAGCTATGGCGGATTCGCCGTAGCGGATATGAATTTGTTAACTGAGACAAACTAAATGGATAAATTCGTGTTTCAGGGGCCAACGAACTCC

MurZ: g e v t i s g a k n a a l p i l f a a l l a e e p v e i q n v p k

601: AGGGCGAAGTCAACAATTTCCGGCGCTAAAAATGCTGCTCTGCTTATCTTTTTTGGCCGCACTACTGGCGGAAGAACCGGTAGAGATCCAGAACGTTCCCGAA

MurZ: l k d v d t s m k l l s q l g a k v e r n g s v h i d a r d v n v

701: ACTGAAAGACGTCGATACATCAATGAAGCTGCTAAGCCAGCTGGGTGCGAAAGTAGAACGTAATGGTTCTGTGCATATTGATGCCCGCGACGTTAAATGTA

MurZ: f c a p y d l v k t m r a s i w a l g p l v a r f g q g q v s l p g

801: TTCTGCGCACCTTACGATCTGGTAAAACCATGCGTGTCTTATCTGGGCGCTGGGGCGCTGGTAGCGCGCTTTGGTCAGGGCAAGTTTCACTACCTG

MurZ: g c t i g a r p v d l h i s g l e q l g a t i k l e e g y v k a s

901: GCGTTGTACGATCGGTGCGCGTCCGGTTGATCTACACATTTCTGGCTCGAACAAATAGGCGCGACCATCAAACCTGGAAGAGGTTACGTTAAAGCTTC

MurZ: v d g r l k g a h i v m d k v s v g a t v t i m c a a t l a e g t

1001: CGTCGATGGTCGTTTGAAGGTGCACATATCGTGATGGATAAAGTCAGCGTTGGCGCAACGGTGCACATCATGTGTGCTGCAACCTGGCGGAAGGCACC

MurZ: t i i e n a a r e p e i v d t a n f l i t l g a k i s g q g t d r i

1101: ACGATTATTGAAAACGACGCGCTGAACCGGAAATCGTCGATACCGCAACTTCTGATTACGCTGGGTGCGAAAATAGCGGTGAGGCACCGATCGTA

MurZ: v i e g v e r l g g g v y r v l p d r i e t g t f l v a a a i s r

1201: TCGTCATCGAAGGTGTGGAACGTTTAGCGCGCGTGTCTATCGCTTCTGGCGGATCGTATCGAAACCGGTACTTCTCTGGTGGCGGGCGGATTCTCTCG

MurZ: g k i i c r n a q p d t l d a v l a k l r d a g a d i e v g e d w

1301: CGGCAAAATTTATCTGCCGTAACCGCGACCCAGATACTCTCGACCGCGTGGCGAAAATCGCTGACGCTGGAGCGGACATCGAAGTCCGGCAAGACTGG

|--overlap-->>

MurZ: i s l d m h g k r p k a v n v r t a p h p a f p t d m q a q f t l l

1401: ATTAGCCTGGATATGCATGGCAAACGTCGAAAGGCTTTAAGTACGTACCTACCGCGCCATCCGGCATTCGCCGACGATATGCAGGCCAGTTCACGCTGT

MurZ: n l v a e g t g f i t e t v f e n r f m h v p e l s r m g a h a e

1501: TGAACCTGGTGGCAGAAGGACCGGTTTATCACCAGAAACGCTTTTGAACCCTTTATGCATGTGCCAGAGCTGAGCCGTATGGCGCGCACGCGCGA

MurZ: i e s n t v i c h g v e k l s g a q v m a t d l r a s a s l v l a

1601: AATCGAAAGCAATACCGTTATTTGTCACGGTGTGAAAACTTTCTGGCGCACAGGTTATGGCAACCGATCTGCGTGCATCAGCAAGCCTGGTGTGGCT

MurZ: g c i a e g t t v v d r i y h i d r g y e r i e d k l r a l g a n i

1701: GGCTGTATTGCGGAAGGACGCGGTTGATGATTTATCACATCGATCGTGGCTACGAACGCATTGAAGACAAACTGCGCGCTTTAGGTGCAATA

MurZ: e r v k g e @

1801: TTGAGCGTGTGAAAGGCGAATAATCGTCT

FIG. 2. Nucleotide sequence in the vicinity of the *murZ* locus and amino acid sequence encoded by the *murZ* gene. Nucleic acid residues 1 through 207 and 1605 through 1829 were sequenced on one strand only. Overlap with the sequence from Choi et al. (4) is indicated. The locations of *Th1000* transposon insertions are underlined, and the six LXXLGA-polar-hydrophobic-polar motifs are boxed.

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

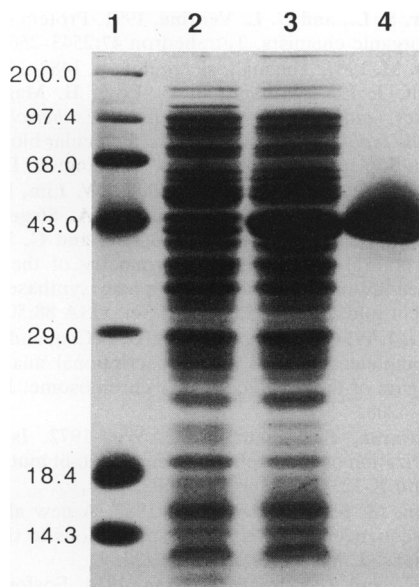


FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel visualized with Coomassie stain showing purification of UDP-GlcNAc enolpyruvate transferase. Lane 1, molecular mass standards in kilodaltons; lane 2, XA90 cells transformed with pKen; lane 3, XA90 cells transformed with the pKen *murZ* overexpression construct after induction with IPTG; lane 4, purified enzyme.

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 the 5'-OH of shikimate-3-phosphate with concomitant release of P_i . 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 MAC6 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, LXXLGA-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.

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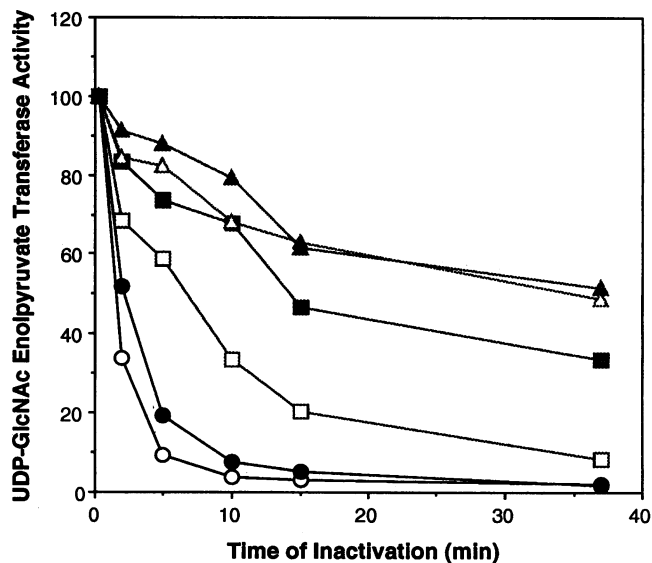


FIG. 4. Purified UDP-GlcNAc enolpyruvate transferase product of the *murZ* gene was inactivated by 0.25 μ M phosphomycin for various times in the presence of various concentrations of UDP-GlcNAc (\circ , 1.000 mM; \bullet , 0.1 mM; \square , 0.01 mM; \blacksquare , 0.001 mM; \triangle , 0.0001 mM). Excess PEP and UDP-GlcNAc were then added, and the activity remaining was assessed by the method of Lanzetta et al. (9) as described in the text. Time-dependent inactivation was enhanced by binding of UDP-GlcNAc. \blacktriangle , H_2O .

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ADDENDUM IN PROOF

Wanke et al. (C. Wanke, R. Falchetter, 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.

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