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MurA (MurZ), the Enzyme That Catalyzes the First Committed Step in Peptidoglycan Biosynthesis, Is Essential in *Escherichia coli*

ERIC D. BROWN,[1] EUGENIO I. VIVAS,[2] CHRISTOPHER T. WALSH,[1] AND ROBERTO KOLTER[2]*

Department of Biological Chemistry and Molecular Pharmacology[1] and Department of Microbiology and Molecular Genetics,[2] Harvard Medical School, Boston, Massachusetts 02115

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The *Escherichia coli* gene *murZ* was recently shown to encode UDP–*N*-acetylglucosamine enolpyruvyl transferase, which catalyzes the first committed step of peptidoglycan biosynthesis (J. L. Marquardt, D. A. Siegele, R. Kolter, and C. T. Walsh, J. Bacteriol. 174:5748–5752, 1992). The map position of *murZ* (69.3 min) differed from that determined for *murA* (90 min), a gene which had been previously proposed to encode the same activity (P. S. Venkateswaran and H. C. Wu, J. Bacteriol. 110:935–944, 1972). Here we describe the construction of a chromosomal deletion of *murZ* and a plasmid containing *murZ* under arabinose control. Growth of cells containing the *murZ* deletion was dependent on the expression of *murZ* from the plasmid. We conclude that *murZ* is an essential gene and encodes the sole UDP–*N*-acetylglucosamine enolpyruvyl transferase of *E. coli*. To simplify the nomenclature, we recommend that *murA* be used to designate the gene at 69.3 min that encodes this activity and that the designation *murZ* be abandoned.

The bacterial cell wall peptidoglycan is an essential cellular component involved in the maintenance of shape and protection from osmotic shock lysis. The *Escherichia coli* peptidoglycan is assembled from a basic building block composed of *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid with an attached pentapeptide (10). The first committed step in peptidoglycan biosynthesis is catalyzed by UDP-GlcNAc enolpyruvyl transferase. Our interest in this enzyme stems from its unusual reaction mechanism (2) involving the transfer of enolpyruvate from phosphoenolpyruvate to the 3-OH of UDP-GlcNAc and from its status as the target of the antibiotic fosfomycin (6).

In 1972, Venkateswaran and Wu reported the isolation of a temperature-sensitive and fosfomycin-resistant mutant of *E. coli* (12, 14). The UDP-GlcNAc enolpyruvyl transferase activity of this mutant was resistant to fosfomycin in vitro. The antibiotic-resistant and temperature-sensitive allele, designated *murA*, was mapped by conjugation to be near the *argECBH* cluster at 90 min (12, 14). We recently cloned an *E. coli* gene encoding a UDP-GlcNAc enolpyruvyl transferase by screening for fosfomycin resistance resulting from the overexpression of the gene on a multicopy plasmid (9). The map position of that gene was determined to be 69.3 min. Because its map position differed from that of *murA*, we designated the gene *murZ*. Taken together, these studies suggested that *E. coli* might contain two genes encoding UDP-GlcNAc enolpyruvyl transferase activities. To test this hypothesis, we set out to construct a null allele of *murZ*. We reasoned that if a second gene encoded a redundant transferase, a strain containing a deletion of *murZ* should be viable.

The strategy for constructing a precise deletion of *murZ* employed a gene replacement protocol originally developed by Hamilton et al. (5) and refined more recently by Link et al. (7) with the development of the knockout plasmid pKO3. Plasmid pKO3 has been designed to replace genomic DNA with mutated chromosomal sequences which have been cloned into the vector. The plasmid carries a temperature-sensitive origin of replication (pSC101-ts) and chloramphenicol resistance so that at the nonpermissive temperature (42°C) and in the presence of the antibiotic, chromosomal integrants can be selected. This results in the generation of a tandem duplication of the wild-type and mutant alleles of the cloned gene with the plasmid sequence in between the two copies. A subsequent shift to the permissive temperature (30°C) selects for the excisants. Depending on which flanking sequence is used in the recombination event, excision results in either restoration of the wild-type gene or creation of a mutant allele in the chromosome. Cells that have lost the plasmid can then be selected because of the presence in pKO3 of the *Bacillus subtilis sacB* gene, whose expression in the presence of sucrose is detrimental to *E. coli* (1). Figure 1A depicts pKO3-ΔZ, which contains a kanamycin resistance cassette in between the *murZ* flanking sequences (denoted L and R). The kanamycin resistance gene makes possible a selection for that excision event generating the mutant allele following a shift to the permissive temperature. The result would be loss of *murZ* through recombination of the cointegrant to yield the Δ*murZ*::*kan* deletion-insertion mutation (Fig. 1C).

In the first attempts to delete *murZ*, wild-type *E. coli* ZK126 was transformed with pKO3-ΔZ, and transformants were grown overnight at 30°C in Luria-Bertani (LB) broth containing kanamycin and chloramphenicol (see Table 1 for strains used in this study). An estimated 10,000 transformants were then plated onto prewarmed LB agar plates containing chloramphenicol and kanamycin and were incubated at 42°C to select for cointegrants. Typically, about 100 colonies were obtained. Fifty of these colonies were streaked onto LB agar containing kanamycin and 5% (wt/vol) sucrose and were incubated at 30°C for 24 h to select for cells in which plasmid excision resulted in the replacement of *murZ* with the mutant allele and loss of the plasmid. However, all of the isolates obtained this way retained both kanamycin and chloramphenicol resistance, indicating retention of plasmid sequences. The retention of chloramphenicol resistance after growth on su-

* Corresponding author. Phone: (617) 432-1776. Fax: (617) 738-7664. Electronic mail address: kolter@mbcrr.harvard.edu.

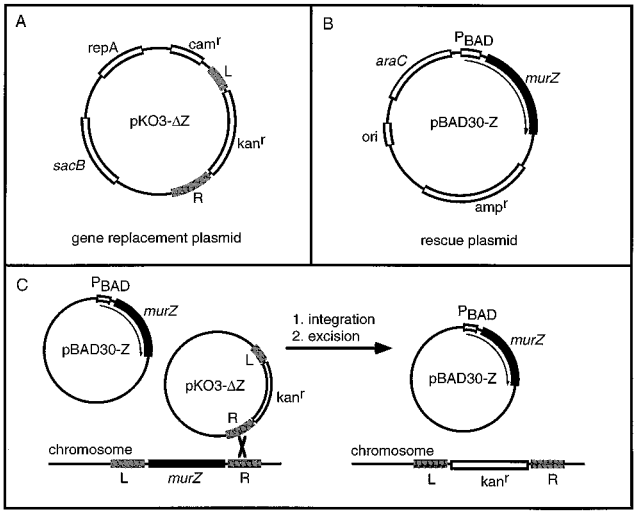

FIG. 1. Strategy for the deletion of the chromosomal copy of *murZ*. pKO3-ΔZ (A) was constructed with pKO3 (7), which was derived from gene replacement vectors developed originally by Hamilton et al. (5). pKO3-ΔZ contains a kanamycin resistance cassette flanked on one side by a sequence identical to 542 bp of the *E. coli* chromosome upstream of and adjacent to the start codon of *murZ* (flank L) and on the other side by a sequence identical to 873 bp immediately downstream of the termination codon of *murZ* (flank R). The flanking sequences were amplified by PCR with Vent DNA polymerase and the template plasmid pJLM2 (8) and the following primers: upstream flank, 5′-CCTTAAGTAAACGACAAAGTGTA-3′ (primer 1) and 5′-*CGAGATCTCGCCCCC*TTAGTTTGTTCTCAGT-3′ (primer 2), and downstream flank, 5′-*GGGGCGAGATCTCG*TAATCGTCTGAGAGCT-3′ (primer 3) and 5′-CCTTAAGAACAGTTAGGTAAAAA-3′ (primer 4) (*Bgl*II sites are underlined, and the complementarity of primers 2 and 3 is shown in italic). The resulting PCR-amplified flanks L and R were then ligated through their complementary sequences by (i) annealing (2 min at 98°C, 10 min at 65°C, and 10 min at 37°C), then (ii) extension with Klenow polymerase for 15 min at 22°C followed by denaturation for 10 min at 75°C, and finally (iii) PCR amplification with Vent DNA polymerase and primers 1 and 4. The resulting L-R fragment (1,443 bp) was then blunt ligated into *Eco*RV-digested pBluescript II SK to create plasmid pBlue-L R. The kanamycin resistance cassette was a 1,706-bp *Bam*HI fragment from mini-Tn*10*kan (13) and was ligated into the unique *Bgl*II site of the vector pBlue-L R to create pBlue-L Kn R. The kanamycin resistance cassette and flanks were then digested from plasmid pBlue-L Kn R (3,188-bp *Sal*I-*Bam*HI fragment) and ligated into *Sal*I-*Bam*HI-digested plasmid pKO3 to produce pKO3-ΔZ. Plasmid pBAD30-Z (B) contained the *murZ* gene under the control of the arabinose promoter P_{bad}. The *murZ* gene (sequence, −19 to stop) was amplified by PCR with Vent DNA polymerase (primers 5′-GCGGCTAGCTAACTGAGAACAAACTAA-3′ and 5′-GCGCAAGCTTCGATTATTCGCCTTTCAC-3′) and blunt ligated into *Eco*RV-digested pBluescript SK to generate plasmid pBlue-Z. Subsequently, the *murZ* gene was digested from pBlue-Z (1,385-bp *Xba*I-*Kpn*I fragment) and ligated into *Xba*I-*Kpn*I-digested pBAD30 (C) (4).

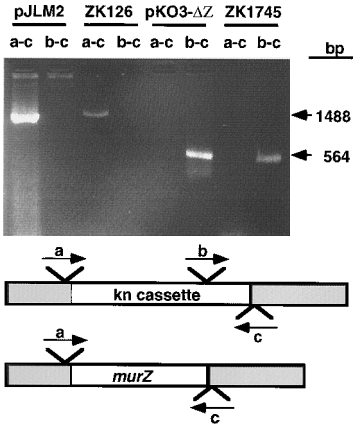

FIG. 2. Verification of gene replacement in the mutant ZK1745. PCR amplifications from purified plasmid DNA and single-colony DNA with primer a (5′-TAACCGCATTCATGCTGTGT-3′), primer b (5′-CACCCCTTGTATTACTGTTTATGT-3′), and primer c (5′-ACAGAACGCAGTTGATGCGTAGCT-3′) are shown. Primers a and c (a-c) and primers b and c (b-c) were used for amplifications from each of the four templates: plasmid pJLM2 (containing the *murZ* gene and flanking DNA), colony ZK126 (wild-type *E. coli*), plasmid pKO3-ΔZ (knockout vector containing the kanamycin cassette and *murZ* flanking sequences; see the legend to Fig. 1), and colony ZK1745 (*E. coli* mutant ZK126 Δ*murZ*::kn/pBAD30-Z).

crose can most easily be explained by spontaneous mutations in *sacB*. These results were most consistent with the hypothesis that *murZ* is essential.

To further test the possibility that *murZ* is essential, the same experiment was carried out in strain ZK126, which carried pBAD30-Z (Fig. 1B), a plasmid containing *murZ* under the control of the arabinose-inducible P_{bad} promoter. The plasmid pBAD30-Z is derived from vector pBAD30, one of a series of pBAD expression vectors which have recently been constructed to aid in studying the phenotypes of null mutations of essential genes (4). Gene replacement experiments with ZK126/pBAD30-Z and pKO3-ΔZ were performed identically as described above, except that integration was at 42°C on LB agar containing ampicillin, chloramphenicol, kanamycin, and 0.2% arabinose. Colonies from the 42°C plates were subsequently streaked on LB agar containing ampicillin, kanamycin, 5% sucrose, and 0.2% arabinose and incubated at 30°C. From the sucrose-containing plates, a number of isolates which were chloramphenicol sensitive and whose growth on LB agar containing kanamycin and ampicillin was dependent on the presence in this medium of 0.2% arabinose were collected. Subsequent P1 transduction of kanamycin resistance from three of the mutants into ZK126/pBAD30-Z or into an Ara mutant bearing the same plasmid (ZK1488/pBAD30-Z) was successful only when transductants were plated on medium containing 0.2% arabinose. Subsequent restreaking of these transductants on either LB or minimal glycerol plates yielded isolates only in the presence of 0.2% arabinose. Thus, viability of the Δ*murZ*::Kan^r insertion-deletion mutant could be maintained only when *murZ* was provided in *trans*.

Figure 2 illustrates the use of PCR to confirm the replacement of *murZ* with the kanamycin resistance cassette in the mutant (ZK1745). Primers a and c were complementary to sequences which flank *murZ* in the wild type (ZK126) and the location of the kanamycin cassette in the mutant ZK1745. Amplification from the *murZ*-containing templates, plasmid pJLM2 (containing cloned *murZ* and flanking sequences) and the ZK126 chromosome, produced fragments of 1,488 bp, as was expected for an intact *murZ*. Amplification with these same primers with either pKO3-ΔZ or the ZK1745 chromosome failed to generate any amplified product (the theoretical product was 1,848 bp). We suspected that the difficulty in amplifying the latter product could be due to a large inverted repeat of 363 bp which was present at the both the 5′ and 3′ ends of the kanamycin resistance cassette. To overcome this

TABLE 1. Bacterial strains

Strain	Description	Source or reference
ZK126	W3110 *tna2* Δ*lacU169*	3
ZK1488	ZK126 Δ*ara*	Lab stocks
ZK1745	ZK126 Δ*murZ*::*kan*(pBAD30-Z)	This work
ZK1746	ZK1488 Δ*murZ*::*kan*(pBAD30-Z)	This work

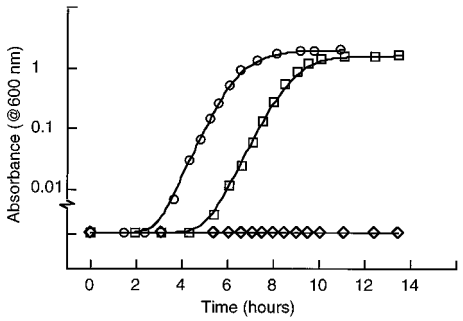

FIG. 3. Growth of the *murZ* deletion (ZK1745) in the presence and absence of arabinose. The growth curves shown are for 100-ml shaking cultures of ZK126 in LB (○), ZK1745 in LB with kanamycin, ampicillin, and 0.2% arabinose (□), and ZK1745 in LB with kanamycin and ampicillin but without arabinose (◇). Prior to the experiment, 2 ml of LB with kanamycin and ampicillin but without arabinose was inoculated with cells from a single colony of ZK1745 (on LB–kanamycin–ampicillin–0.2% arabinose agarose plates), and the cells grew overnight to an OD_{600} of only 0.05. For the growth curves of ZK1745 with and without arabinose (□ and ◇), 0.75 ml of that overnight culture was the inoculum. In the case of ZK126, an overnight culture was diluted to an OD_{600} of 0.05 and 0.75 ml of the diluted culture served as the inoculum.

problem, we synthesized a primer (b) which annealed upstream of the 3′ repeat. The reasoning was that amplification with primers b and c would produce a shorter, more easily amplified fragment which would circumvent any intrastrand structure present as a result of the inverted repeats. Indeed, amplification with primers b and c from plasmid pKO3-ΔZ and from the mutant generated fragments consistent with the predicted size of 564 bp and confirmed the replacement of *murZ* with the kanamycin cassette in mutant ZK1745. We note also that Southern analysis of *Sma*I-*Xba*I digests of ZK126/pBAD30Z and ZK175 with the L Kn R fragment being used as a probe (see the legend to Fig. 1) revealed a single 16.6-kb fragment in the wild-type genome but demonstrated two fragments with sizes of 7.3 and 9.5 kb in the mutant (data not shown). Like that of the PCR, the Southern blot result is diagnostic of the replacement of *murZ* by the kanamycin cassette since only the kanamycin resistance gene is cut by the restriction enzymes used (single *Sma*I site).

Having established the arabinose-dependent growth of mutant ZK1745 on solid media, we also tested its ability to grow in LB broth in either the presence or absence of arabinose. We consistently noted that the inoculation of LB broth without arabinose with cells from a colony of ZK1745 growing on an arabinose-containing plate produced at least a small amount of growth, presumably because of the time necessary for the cells to deplete the functional MurZ carried over in the inoculum. Figure 3 shows the time course of growth after inoculation of 100 ml cultures (LB broth containing kanamycin and ampicillin with or without 0.2% arabinose) with 0.75 ml of an overnight liquid culture (LB broth containing kanamycin and ampicillin but no arabinose) of ZK1745 which had been picked from an arabinose-containing plate. Most striking is the complete lack of growth of the culture without arabinose, even after the arabinose-containing culture reached stationary phase (optical density at 600 nm [OD_{600}], 1.7). In fact, subsequent addition of arabinose to the culture without arabinose enabled that flask to reach an equivalent OD_{600} within 14 h (data not shown). We believe that the apparent stasis of the culture without arabinose is the result of very slow growth due to the extremely low expression of *murZ*, in contrast to the well-documented bactericidal and lytic outcome of treatment with the antibiotic fosfomycin (6). Also shown in Fig. 3 is the time course of the growth of wild-type ZK126 in LB broth after inoculation with 0.75 ml of an overnight culture which had been diluted to an OD_{600} equivalent to that of the ZK1745 inoculum (OD_{600} = 0.05). While the lag phases of growth for the wild type (2 h) and for ZK1745 grown in the presence of arabinose (4 h) differed slightly, growth rates and final optical densities were not dissimilar, indicating that expression of *murZ* from P_{bad} resulted in only slight growth differences compared with the growth of the wild-type control. Indeed, the differences in lag may very well have been due to differences in the amounts and viabilities of inocula from the mutant and wild-type cells.

All of these results provide strong evidence in support of the hypothesis that *murZ*, located at 69.3 min, is an essential gene and therefore call into question the existence of a second UDP-GlcNAc enolpyruvyl transferase coded for by a gene located near 90 min (*murA*) (12, 14). Consistent with this conclusion is our inability to find any candidate open reading frames for a second UDP-GlcNAc enolpyruvyl transferase by using the MurZ sequence as a probe to search the available translated sequences of *E. coli*, which include the complete 80- to 100-min region. The question remains as to why the original *murA* mutant, which was both resistant to fosfomycin and temperature sensitive, was mapped to 90 min. Preliminary studies in our laboratory with this mutant strain suggested that it has multiple mutations: temperature sensitivity mapped near 90 min, but fosfomycin resistance mapped near 70 min (11). Thus, it is very likely that *E. coli* contains a single gene which encodes UDP-GlcNAc enolpyruvyl transferase, which is located at 69.3 min, and which we recently designated *murZ*. To simplify the nomenclature, we recommend that *murA* be used to designate the gene at 69.3 min that encodes this activity and that the designation *murZ* be dropped.

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