Alteration of a single amino acid residue reverses fosfomycin resistance of recombinant MurA from *Mycobacterium tuberculosis*

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*Mycobacterium tuberculosis* has innate resistance to a range of broad-spectrum antimicrobial agents. This may in part reflect the relative impermeability of the mycobacterial cell wall, but additional specific mechanisms may also be important. In the case of fosfomycin, it has been suggested that a key difference in the active site of the *M. tuberculosis* MurA enzyme might confer resistance. In *Escherichia coli*, fosfomycin covalently binds to a cysteine normally involved in the enzymic activity, while protein alignments predict an aspartate at this position in the *M. tuberculosis* MurA. In the present study, it is demonstrated that the wild-type *M. tuberculosis* MurA is indeed resistant to fosfomycin, and that it becomes sensitive following replacement of the aspartate residue in position 117 by a cysteine. In addition, the study illustrates the use of an inducible expression system in mycobacteria to allow functional characterization of an *M. tuberculosis* enzyme that is unstable during constitutive expression.

**Keywords:** acetamidase, fosfomycin, MurA, mycobacteria, tuberculosis

INTRODUCTION

The emergence of isolates of *Mycobacterium tuberculosis* resistant to currently available therapies (Pablos-Mendez et al., 1998) highlights the need for development of new drugs to combat an infection that claims more human life than any other single pathogen. The current panel of antimycobacterial agents is restricted to only a handful of compounds, largely because mycobacteria display an innate resistance to many of the common antimicrobials. This resistance is generally ascribed to the relative impermeability of the mycobacterial cell wall, but the identification of efflux pumps and of enzymes capable of drug modification (Takiff et al., 1996; Ainsa et al., 1997) suggests that additional resistance mechanisms may also be important. By understanding the mechanisms underlying the innate resistance of *M. tuberculosis* to common antimicrobial agents, it may be possible to design modifications, or novel drug combinations, that would allow their clinical use in tuberculosis therapy.

Fosfomycin is a broad-spectrum antibiotic produced by some strains of *Streptomyces* (Christensen et al., 1969; Hendlin et al., 1969). Its mechanism of action was first described by Kahan et al. (1974), who showed that it inhibits the enzyme UDP-N-acetylglucosamine enolpyruvyl transferase (MurA), which transfers the enolpyruvyl moiety of phosphoenolpyruvate to the 3′ hydroxyl group of UDP-N-acetylglucosamine, the first committed step in bacterial peptidoglycan biosynthesis. Investigation of the detailed mechanism of *Escherichia coli* MurA revealed that fosfomycin irreversibly inactivates MurA by forming a covalent adduct with a critical cysteine residue in the active site of the enzyme (Kahan et al., 1974; Skarzynski et al., 1996).

Resistance to fosfomycin is commonly by decreased uptake of the drug, although drug inactivation by a plasmid-encoded glutathione transferase has also been reported (Arca et al., 1997). Fosfomycin has no activity against *M. tuberculosis*, and Kim et al. (1996) noted that, in the sequence of the *M. tuberculosis murA* homologue, the key active site cysteine (amino acid 117

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in the *M. tuberculosis* sequence) is replaced by an aspartic acid residue. They went on to show that replacement of cysteine by aspartate in *E. coli* MurA resulted in retention of enzyme activity, but conferred resistance to fosfomycin. They postulated that the presence of aspartate in place of cysteine in the MurA enzyme might account for the innate fosfomycin resistance of *M. tuberculosis*.

The aim of the present study was firstly to determine whether or not *M. tuberculosis* MurA is inhibited by fosfomycin, and secondly to assess the effect of substitution of cysteine for aspartate-117 on fosfomycin susceptibility of *M. tuberculosis* MurA. In addressing these questions, we encountered difficulties in expression of recombinant *M. tuberculosis* MurA in a functionally active form. It formed insoluble products in a range of *E. coli* expression systems and, although it was soluble in mycobacterial systems, expression was rapidly lost during subculture. We report on the use of an inducible expression system in *Mycobacterium* to overcome problems associated with stable expression of *M. tuberculosis* MurA, and demonstrate that, as in *E. coli* MurA, residue 117 is a critical determinant of fosfomycin susceptibility.

**METHODS**

Cloning of the *M. tuberculosis* murA gene. For expression analysis in *E. coli*, the *M. tuberculosis* murA gene was amplified from *E. coli* (gift from Dr J. Colston, NIMR, Mill Hill, London) (Kempesell et al., 1992) using primers MurA5′ and MurA3′ (Table 1) to generate clone MurA2T6. MurA5′ is homologous to the 5′-terminus of the murA gene upstream sequence and also spans the initiation codon, substituting nucleotides −2 to +1 with CCA (depicted in bold italics in Table 1) to generate an ATG start codon and unique NcoI cutting site at the 5′-terminus of the coding region. MurA3′ is homologous to the 3′ proximal downstream sequence with an A for C substitution at nucleotide 12 (depicted in bold italic) to generate a unique NdeI site downstream of the termination codon. After amplification the fragment was cut with NcoI and NdeI and cloned into these sites in expression vector pET-19b (Novagen, NBL Gene Sciences) to yield pMurA2T6, and the sequence was verified. This cloning procedure placed the modified initiation codon downstream of the T7lac promoter and an *E. coli* ribosome-binding site. All attempts at cloning murA into the vector pTrc99A (Amersham Pharmacia Biotech) were unsuccessful, probably due to the ‘leaky’ nature of this promoter.

For antibody production and mycobacterial expression, the murA gene was amplified from *M. tuberculosis* H37Rv chromosomal DNA by PCR using Deep Vent Polymerase (New England Biolabs) with oligonucleotide primers MUR-1 and MUR-2 (Table 1). The PCR product was cloned using the pCR-Script cloning kit (Stratagene), and subsequently excised as a BamHI–HindIII fragment for subcloning in pQE31 (Qiagen) (for production of histidine-tagged recombinant in *E. coli*), pSMT3 (to generate pSM-murA), p19Kpro (p19-murA) and pSODIT-2. To align the BamHI–HindIII fragment in-frame with the start codon in pSODIT-2, the initial construct was cleaved with BamHI, treated with mung bean nuclease, and religated to produce pSOD-murA. To clone murA in pACE (in which HindIII is not a unique site), a BamHI–AlwNI fragment was excised from p19-murA and cloned in pACE digested with the same enzymes (to generate pACE-murA). Plasmids were constructed and amplified in *E. coli* using standard procedures and, where appropriate, introduced into *Mycobacterium smegmatis* by electroporation as described previously (Garbe et al., 1994). To analyse mutations occurring during subculture, plasmids were isolated from *M. smegmatis* cells incubated for 1 h with 10 mg lysozyme ml⁻¹ in Tris/EDTA buffer followed by plasmid purification using a commercial kit (Qiagen).

A mutant form of *M. tuberculosis* murA with cysteine in place of aspartate in position 117 (D117C) was prepared by using the complementary primers MUR-3 and MUR-4, covering codon 117. Two independent amplifications using primer sets MUR-1 + MUR-4 and MUR-2 + MUR-3 produced two fragments of the murA gene. After removal of remaining primers, the two PCR products were pooled and the full-length D117C murA gene was amplified by PCR using the outer primers. The PCR product was cloned in pCR-Script and then subcloned in pACE. Sequence determination using fluorescent dideoxynucleotides on an ABI 310 analyser con-

<table>
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<th>Sequence</th>
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<tr>
<td>19-1</td>
<td>GCTCTAGACGGTTTTGTGTTCCATCG</td>
</tr>
<tr>
<td>19-2</td>
<td>CCGGATCCCTTGCTCCTTTGCTCGT</td>
</tr>
<tr>
<td>SOD-1</td>
<td>GCTCTAGACACGGCTTGGGGGCGTCCTG</td>
</tr>
<tr>
<td>SOD-2</td>
<td>GGGGATCCACGCCCATTCTCTTCATCGAT</td>
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<td>ACE-1</td>
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<td>ACE-2</td>
<td>GGGATCTGGAATTTACGCAGCTTCT</td>
</tr>
<tr>
<td>MUR-1</td>
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<tr>
<td>MUR-2</td>
<td>AAGAATCTAAGCGATACCCGTTT</td>
</tr>
<tr>
<td>MUR-3</td>
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<tr>
<td>MUR-4</td>
<td>CCGATCGGCGCAACCGGCC</td>
</tr>
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<td>MurA5′</td>
<td>TGACAAGCAGCTATATAGTAGATCTATATG</td>
</tr>
<tr>
<td>MurA3′</td>
<td>GCGACGCTACATGATGATAGATGACCGA</td>
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**Table 1.** Primers used for PCR amplification

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firmed that the construct encoded murA with the desired single change at position 117.

**Mycobacterial expression vectors.** Mycobacteria–*E. coli* shuttle vectors were constructed by modification of the previously described hygromycin-resistance vector p16R1 (Garbe et al., 1991). pOLYG, a p16R1 derivative with a multiple cloning site, and pSMT3, an expression plasmid containing the promoter region from *M. tuberculosis* hsp60, have been described previously (O’Gaora et al., 1997). To generate p19Kpro, a 195 bp region upstream of the 19 kDa antigen gene (Ashbridge et al., 1989) was amplified from *M. tuberculosis* H37Rv chromosomal DNA by PCR using Deep Vent Polymerase (New England Biolabs) with oligonucleotide primers 19-1 and 19-2 (Table 1). The PCR product was digested with XbaI and BamHI, and cloned in pOLYG digested with the same enzymes. Similarly, pSODIT-2 was constructed by PCR amplification of a 193 bp fragment from *M. tuberculosis* sodA (Zhang et al., 1991) using primers SOD-1 and SOD-2, followed by cloning in pOLYG. pACE was constructed by PCR amplification of a 3 kb region upstream of the acetamidase gene of *M. smegmatis* (Mahenthiralingam et al., 1993; Parish et al., 1997) using primers ACE-1 and ACE-2.

**Expression of MurA in *M. smegmatis*.** Single hygromycin-resistant colonies obtained after electroporation of *M. smegmatis* were inoculated into 5 ml Luria–Bertani (LB) medium, or minimal medium containing 0·2% (w/v) succinate as carbon source, and cultured for 1–3 d with shaking at 37 °C. Minimal medium was prepared by dissolving the following in 1 l distilled water: 4 g NaCl, 0·2 g MgSO$_4$, 2 g KH$_2$PO$_4$, 2 g (NH$_4$)$_2$HPO$_4$, 2 g Na-succinate, 0·05% Tween 80, pH 7·2. After autoclaving, 2 ml filter-sterilized trace element solution was added, containing (l$^{-}$1) 40 mg ZnCl$_2$, 200 mg (NH$_4$)$_2$Fe(SO$_4$)$_2$·6H$_2$O, 10 mg CuSO$_4$·5H$_2$O, 10 mg MnCl$_2$·4H$_2$O, 10 mg borax and 10 mg ammonium molybdate. Samples were then checked for expression (see below) or mixed 1:1 with 50% glycerol and frozen at −80 °C. To analyse the effect of subculture, 10 ml LB medium (pSOD-murA) or minimal medium (pACE-murA) was inoculated from frozen stock and incubated with shaking at 37 °C till the cultures reached stationary phase (2–3 d). Ten microlitres was used to inoculate 10 ml fresh medium, and incubation and subculture steps were repeated. To analyse MurA expression in the inducible vector system, pACE constructs were grown to stationary phase in minimal medium, diluted 1/100 in fresh medium and grown overnight. Acetamide was added to a final concentration of 0·2% (w/v), and cultures were grown for a further 24 h with shaking at 37 °C.

**Production of antiserum and Western blotting.** *M. tuberculosis* MurA was expressed in *E. coli* using the pQE31 vector (Qiagen). Cultures were induced by IPTG according to the manufacturer’s recommended procedures, generating insoluble protein which was denatured in the presence of urea and purified by nickel affinity chromatography (Qiagen). Subsequent removal of the urea by dialysis resulted in precipitation of the recombinant protein, which was emulsified with Freund’s incomplete adjuvant and used to immunize BALB/c mice. Mice received two intraperitoneal injections of 50 µg protein 2 weeks apart, with subsequent boosting with protein without adjuvant.

To prepare *M. smegmatis* samples for analysis of MurA expression, bacteria from 10 ml cultures were harvested by centrifugation, suspended in PBS and disrupted by sonication [Soniprep 150 (MSE) for 10 s at an amplitude of 8 microns]. Cell debris was removed by centrifugation for 5 min in a microfuge and protein levels in soluble extracts were determined using the Coomassie Plus Protein Assay (Pierce). Soluble extracts (5 µg protein per lane) were fractionated by SDS-PAGE and transferred to nitrocellulose membranes for Western blotting. Blots were developed using the monoclonal antiserum raised against *M. tuberculosis* MurA at a dilution of 1:1000. Antibody binding was visualized using peroxidase-conjugated anti-mouse antibody, with subsequent incubation with SuperSignal (Pierce) and autoradiography.

**Assay of MurA activity.** To assay MurA activity, pACE constructs in *M. smegmatis* were grown with or without acetamide induction as described above. After the final incubation, cells were harvested by centrifugation, washed in 50 mM Tris/Cl (pH 7·5) with 2 mM DTT, and lysed by sonication as above. Cell debris was removed by centrifugation for 5 min in a microfuge, and the resulting supernatant was subjected to gel filtration using a PD10 column (Pharmacia) to remove small molecules that may interfere with enzyme assays.

The assay mixture (total volume 50 µl) contained buffer (50 mM Tris/Cl, pH 7·5, 2 mM DTT), UDP-N-acetylglucosamine (10 mM) and bacterial extract (5–15 µg protein). After 15 min at 37 °C, the reaction was started by addition of 5 µl phosphoenolpyruvate (to a final concentration of 1 mM), and was stopped after 1 h by addition of 200 µl colour reagent [1% ammonium molybdate, 12·5% HCl, 0·15% malachite green (Itaya & Ui, 1966)]. Results are expressed as the $A_{5179}$ corrected for the background reading observed in the absence of UDP-N-acetylglucosamine. Where appropriate, fosfomycin (Sigma) was added during the initial incubation period at concentrations ranging from 30 µM to 10 mM. Appropriate dilutions were added to the reactions instead of water to reach the required concentration without altering the final volume.

**RESULTS**

**Cloning and expression of *M. tuberculosis murA* in *E. coli***

The *M. tuberculosis murA* gene was located when a partial ORF having homology to the Y region of *E. coli* murA was identified upstream of the single rRNA operon (Kempshall et al., 1992) using the tFASTA GCG search program (University of Wisconsin). ‡EMBL3 clones containing the entire *M. tuberculosis* rRNA operon had been isolated during the previous study and so a 3·1 kb EcoRI fragment from one such clone, ‡EMBL3/TB3, was cloned into pUC19. The remainder of the *murA* gene was sequenced by extension of an oligonucleotide primer complementary to the previously determined sequence, then ‘walking’ until the sequence had been unambiguously determined on both strands. These data were submitted to EMBL (accession no. X96711) and subsequently confirmed when the complete genome sequence was published (Cole et al., 1998).

**Expression of *M. tuberculosis* MurA protein from clone pMurA2T6.** All attempts to express native soluble MurA in *E. coli* transformed with pMurA2T6 were unsuccessful. A range of growth conditions, rich and minimal media, and IPTG induction conditions were tested. In each case, although expression of MurA protein was visible on SDS-PAGE, the protein was in the insoluble
Cloning of *M. tuberculosis* murA in mycobacterial expression vectors. The *murA* gene of *M. tuberculosis* was expressed as a transcriptional or translational fusion in vectors constructed by insertion of promoter regions from four different mycobacterial genes in plasmids derived from p16R1 (Garbe *et al.*, 1994). In addition to the BamHI cloning site (underlined), pSMT3, p19Kpro and pSODIT-2 have unique PstI, EcoRV, HindIII and ClaI cloning sites. pACE has unique BamHI and ClaI sites. The different N-terminal predicted protein sequences, starting from either an ATG or GTG initiation codon provided by the vector, or the ATG of the cloned *murA*, are shown. Met-Ala-Glu (shown in bold) are the first three amino acids of *M. tuberculosis* MurA.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Cloning Site</th>
<th>Protein Name</th>
<th>Sequence</th>
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<tr>
<td>pSM-murA</td>
<td>404 bp upstream</td>
<td>MetAlaLysThrIleAlaAspProMetAlaGlu(MurA)</td>
<td>ATGGCCAAGACAATGCGATCCCATGGGCCGA</td>
</tr>
<tr>
<td>p19-murA</td>
<td>195 bp upstream</td>
<td>MetAlaGlu(MurA)</td>
<td>GGATCCATGGGCCGA</td>
</tr>
<tr>
<td>pSOD-murA</td>
<td>193 bp upstream</td>
<td>ValGlySerMetAlaGlu(MurA)</td>
<td>GGTGGAATCCATGGGCCGA</td>
</tr>
<tr>
<td>pACE-murA</td>
<td>3 kb upstream</td>
<td>MetAlaGlu(MurA)</td>
<td>GGATCCCGATGGGCCGA</td>
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Fig. 1. Cloning of *M. tuberculosis* murA in mycobacterial expression vectors. The *murA* gene of *M. tuberculosis* was expressed as a transcriptional or translational fusion in vectors constructed by insertion of promoter regions from four different mycobacterial genes in plasmids derived from p16R1 (Garbe *et al.*, 1994). In addition to the BamHI cloning site (underlined), pSMT3, p19Kpro and pSODIT-2 have unique PstI, EcoRV, HindIII and ClaI cloning sites. pACE has unique BamHI and ClaI sites. The different N-terminal predicted protein sequences, starting from either an ATG or GTG initiation codon provided by the vector, or the ATG of the cloned *murA*, are shown. Met-Ala-Glu (shown in bold) are the first three amino acids of *M. tuberculosis* MurA.

Cloning and expression of *M. tuberculosis* murA in *M. smegmatis*

Mycobacterial expression vectors. To test for expression in the rapid growing mycobacterial host strain *M. smegmatis*, the *M. tuberculosis* murA gene was cloned under the control of different promoters in a set of three expression vectors (Fig. 1). All three vectors were derived from p16R1 (Garbe *et al.*, 1994), which encodes resistance to hygromycin, and has replication origins suitable for maintenance in *E. coli* and in mycobacteria. In addition, pSMT3 contains a 389 bp fragment corresponding to the promoter and first six amino acids of *M. tuberculosis* groEL (*hsp60*) (O’Gaora *et al.*, 1997). p19Kpro contains a 195 bp fragment from the promoter region of the *M. tuberculosis* 19 kDa antigen (Ashbridge *et al.*, 1989), and pSODIT-2 has a 193 bp promoter region from the *M. tuberculosis* superoxide dismutase (*sodA*) gene (Zhang *et al.*, 1991), engineered to include a start codon and two additional amino acids (i.e. Met-Gly-Ser) fused to the N-terminus of expressed proteins. Clones containing the *M. tuberculosis* murA gene in the three different expression vectors were named pSM-murA, p19-murA and pSOD-murA, respectively.

Hygromycin-resistant colonies obtained as a result of electroporation of *M. smegmatis* with each of the MurA recombinants were inoculated into 5 ml medium, cultured until stationary phase was reached (1–3 d), and then analysed by SDS-PAGE and Western blotting with monovalent antiserum raised against *M. tuberculosis* MurA. Each of the constructs gave a similar level of expression of a strongly antibody-reactive band with a molecular mass of approximately 44 kDa (Fig. 2). Under the conditions used, background reactivity was not observed in extracts from *M. smegmatis* transformed with vector alone (Fig. 2, lane 1). Reactivity with a similar-sized band corresponding to the endogenous *M. smegmatis* MurA was observed using higher antibody concentrations or following prolonged exposure of blots. The recombinant protein was not visible as a distinct band on gels stained with Coomassie blue, though this may be because the region around 44 kDa already contains several highly expressed proteins. While initial results with mycobacterial expression systems were encouraging, attempts to scale up growth of the recombinants were unsuccessful, with MurA expression being rapidly lost during subculture.

![Fig. 2. Expression of *M. tuberculosis* MurA in constitutive expression vectors. Extracts from *M. smegmatis* cultures grown from individual colonies obtained after electroporation with pOLYG (vector control) (lane 1), pSOD-murA (lane 2), pSM-murA (lane 3) and p19-murA (lane 4) were analysed by Western blotting with an antiserum to MurA. A single antibody-reactive band with molecular mass of 44 kDa was seen in recombinants expressing the murA gene.](image-url)
The effect of subculture was examined in detail in the case of recombinant MurA expressed under control of the SOD promoter. Four independent clones of *M. smegmatis* transformed with pSOD-murA were selected following electroporation and subsequently passed five times, diluting cultures 1/1000 in fresh medium each time. Samples from the first, third and fifth passage were analysed for MurA expression by Western blotting (Fig. 3). For each clone, expression dropped rapidly, and was undetectable in samples from the fifth passage. To determine whether loss of expression was the result of changes affecting the host cell or the cloned gene, plasmids were prepared from each of the fifth passage samples and amplified in *E. coli*. Analysis of the resulting plasmids by restriction enzyme digestion revealed a series of different profiles indicative of modifications affecting the cloned gene (data not shown). On reintroduction into *M. smegmatis*, there was no expression of MurA from altered plasmids. To determine if loss of expression was due to mutations in the promoter, the partial sequence of these plasmids was determined using the SOD-1 oligonucleotide as primer. No changes were found in any of the promoters, although alterations were found in the 5' end of the *murA* gene. There was a 17 bp deletion of nucleotides 5–21 of *murA* in one plasmid and a 34 bp duplication of nucleotides 53–86 in another. Both resulted in frameshift mutations. The loss of expression (and change in restriction profiles) for the other clones was not further analysed. In summary, MurA expression was rapidly lost during subculture of *M. smegmatis* clones as a result of a variety of modifications to the plasmid-encoded gene.

**Inducible expression of *M. tuberculosis* murA in *M. smegmatis***. In order to circumvent problems associated with constitutive expression of MurA, the *M. tuberculosis* gene was cloned in a fourth mycobacterial expression vector, pACE. pACE is similar to the other vectors described above, but with expression of inserted genes placed under the control of a 3 kb fragment derived from the upstream region of the *M. smegmatis* acetylomai-dase gene (Mahenthiralingam et al., 1993; Parish et al., 1997). Acetamidase expression is strongly induced by addition of acetamide to the growth medium. Western blot analysis of freshly isolated *M. smegmatis* transformants grown in the presence of acetamide revealed a level of expression of MurA similar to that obtained using the constitutive expression vectors (Fig. 4). Again, recombinant MurA was not detected as a distinct band by Coomassie blue staining; although, as previously reported (Mahenthiralingam et al., 1993), acetamidase was clearly seen as a major protein in induced cultures. A lower level of MurA expression was detectable in the absence of acetamide induction (Fig. 4). In marked contrast to results with the constitutive expression vectors, however, expression of pACE-murA was maintained during subculture in the absence of inducing agent (Fig. 4). Thus, using the inducible pACE system, we were able to obtain stable expression of *M. tuberculosis* MurA at a level suitable for functional analysis.

**Activity of *M. tuberculosis* MurA and effect of fosfomycin**

Western blot comparison of *M. smegmatis* pACE-murA transformants with vector controls suggested that expression of the recombinant enzyme was at a level 10–20-fold higher than that of the endogenous *M. smegmatis* MurA. Similarly, measurement of phosphatase...
with higher MurA activity than vector control samples, although the activity was typically around twofold lower than that observed using the wild-type gene (see Fig. 5a). In this case, however, preincubation with 10 mM fosfomycin resulted in a marked inhibition of enzyme activity (Fig. 5a). Fig. 5(b) shows a titration of the inhibitory effect of fosfomycin on the D117C mutant form of M. tuberculosis MurA.

**DISCUSSION**

Stable transformation of mycobacteria with plasmid constructs was first reported by Jacobs et al. (1987). Expression vectors initially focused on use of promoters from genes encoding the well-characterized mycobacterial heat-shock proteins (Stover et al., 1991), although several other promoters have been used in subsequent experiments. Results with the MurA enzyme, as well as other experience in our laboratory, suggest that the level of expression obtained in recombinant mycobacterial constructs can be influenced as much by the nature of the cloned gene as by the particular promoter used: the initial level of M. tuberculosis MurA expression in M. smegmatis was similar with each of the four promoters tested. The level of MurA expression obtained using the pACE vector was considerably lower than that of acetamidase in the same cells, in spite of the fact that the MurA construct is present in multiple copies on the plasmid vector. Factors underlying the difference in expression level might include: (a) sequences outside of the 3 kb upstream region might play some role in regulation; (b) a potential stem–loop structure 175 bp downstream of the murA start codon may influence translation or mRNA stability; (c) differences in codon usage might affect translation efficiency – GCT (Ala) and TTG (Leu) codons are used nine times and 14 times, respectively, in the murA gene but not at all in the acetamidase gene, for example, while TCC (Ser) is used six times in acetamidase but not in murA. Further investigation of these effects may be useful in developing improved mycobacterial expression systems (Andersson & Sharp, 1996).

Rapid loss of expression of genes cloned under control of constitutively active promoters in mycobacterial plasmid vectors has previously been reported by several authors (Boshoff & Mizrahi, 1998; Kumar et al., 1998; Prammananan et al., 1999). Possible approaches to overcome this problem include the use of recombination-deficient host strains (Prammananan et al., 1999) and the use of regulated promoters. Inducible promoters have been widely used in development of recombinant expression systems in E. coli. In mycobacteria, emphasis on use of expression technology to construct recombinant BCG vaccines has stimulated a search for promoters that are induced during the process of infection in vivo, but less effort has been invested in development of inducible systems suitable for work with mycobacteria in vitro. The potential utility of such systems is illustrated in the present study using the acetamidase promoter; to our knowledge, the only

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![Graph](image)

**Fig. 5.** Enzyme activity of M. tuberculosis MurA expressed in M. smegmatis. (a) Extracts from M. smegmatis clones transformed with pACE (vector control), pACE-murA or pACE-D117C were assayed for release of phosphate during incubation with UDP-N-acetylglucosamine and phosphoenolpyruvate. Filled bars show the effect of addition of 10 mM fosfomycin; open bars show the control. Error bars represent standard deviation between triplicates in a single experiment. There was significant inhibition of D117C activity in the presence of fosfomycin (P = 0.006); fosfomycin had no significant effect on activity of the pACE-murA (P = 0.25) or of the M. smegmatis activity in the vector control (P = 0.38). (b) Extracts from M. smegmatis::pACE-D117C were incubated with varying concentrations of fosfomycin prior to assay of MurA activity. Preincubation with 1 mM fosfomycin resulted in almost complete loss of enzyme activity.
system available for convenient in vitro induction of gene expression in mycobacteria (Mahenthiralingam et al., 1993; Parish et al., 1997; Triccas et al., 1998). With the availability of the complete genome sequence of M. tuberculosis (Cole et al., 1998) it is possible to identify a series of potential drug targets, and there is a need for simple expression systems to allow experimental testing of functional activities predicted on the basis of homology searches. It is likely that other key enzymes will resemble MurA in posing problems when they are constitutively expressed in a functionally active form; inducible expression systems provide an important solution to these problems.

Analysis of the functional activity of M. tuberculosis MurA fully supports the hypothesis put forward by Kim et al. (1996), in that the wild-type enzyme is completely resistant to fosfomycin, and substitution of cysteine at position 117 confers susceptibility. The evolutionary pathway leading to a difference in sequence at position 117 between different bacteria is unknown. M. smegmatis murA resembles the M. tuberculosis gene in having aspartate at position 117 (M. J. Everett & K. Duncan, unpublished data), and it will be of interest to analyze the sequence in other bacterial genera. While results from the present study clearly demonstrate resistance of M. tuberculosis MurA to fosfomycin in cell-free assays, they do not exclude the possibility that other factors – including permeability – could contribute to drug resistance in whole bacilli. Application of gene replacement techniques (Bardarov et al., 1997; Pelicic et al., 1997) to exchange the mutant D117C gene for the wild-type murA in intact mycobacteria provides an experimental strategy to evaluate this possibility. If the alteration in MurA structure is confirmed as the key factor in innate resistance, fosfomycin derivatives designed to bind to a MurA active site containing an aspartate rather than a cysteine residue might represent a route to production of novel drugs active against bacteria, such as the mycobacteria, which express an aspartyl-MurA.

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of expression vectors for use in multiple mycobacterial species. 

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