

Comparison of the Essential Cellular Functions of the Two *murA* Genes of *Bacillus anthracis*[∇]

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Targeted antisense and gene replacement mutagenesis experiments demonstrate that only the *murA1* gene and not the *murA2* gene is required for the normal cellular growth of *Bacillus anthracis*. Antisense-based modulation of *murA1* gene expression hypersensitizes cells to the MurA-specific antibiotic fosfomycin despite the normally high resistance of *B. anthracis* to this drug.

Many bacterial species rely on a cell wall to maintain their structural integrity in a typical nonisotonic environment. Antibiotics such as β -lactams and glycopeptides either inhibit or are poor substrates for the final transpeptidation step of cell wall biosynthesis, resulting in a weakening of the cell wall, followed by rapid lysis and death. These drugs have been a popular mainstay in the treatment of bacterial infections at least in part due to this powerful bactericidal effect. However, there has been a significant increase in the incidence of resistance to these drug classes among important bacterial pathogens. Even so, cell wall biosynthesis remains a valid target for novel antibiotic development, especially for agents that can specifically inhibit any one of the series of essential enzymatic functions involved in assembly of the peptidoglycan.

The first committed step in peptidoglycan biosynthesis is catalyzed by UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA). MurA enzyme has been shown to be inhibited by fosfomycin (5, 7, 13, 19, 20). Previous studies have demonstrated that fosfomycin forms a covalent bond with the active site Cys115 residue of the *Escherichia coli* and *Enterobacter cloacae* MurA enzymes (5, 7, 13, 19, 20). The general use of fosfomycin has been limited by relatively low potency, as well as by bacterial resistance that, in some instances, has been traced to membrane-bound antiporter proteins. For example, *B. anthracis* demonstrates a high degree of intrinsic fosfomycin resistance that may be due to both endogenous and phage-encoded genes (18). Nevertheless, fosfomycin exhibits a bactericidal effect and has been shown to be strongly synergistic with β -lactam antibiotics (4, 22). For these reasons, the development of a novel MurA-specific antibiotic with improved properties would be highly desirable.

One potential drawback of MurA as a novel antibiotic target is the presence of two separate genes, *murA1* and *murA2*, that encode for proteins with the same enzymatic activity in gram-positive pathogens such as *Staphylococcus aureus* and *Streptococcus pneumoniae*. In the latter organism, it was recently shown that mutagenic disruption of either *murA1* or *murA2* did

not significantly alter cell growth, while cells could not survive the removal of both genes (6). Among gram-positive species, the two *murA* homologs are no more than 60% identical at the amino acid level. Differences in the active site would make it difficult to develop a MurA-specific antibiotic that effectively inhibits both enzymes. Like other gram-positive bacteria, *Bacillus anthracis* also contains two *murA* gene homologs (16). The *murA1* gene (BAS5137) is predicted to be the most distal gene in a three-gene operon consisting of two proximal conserved hypothetical genes (BAS5138 and BAS5139) (1). The *murA2* gene (BAS5183) is predicted to be located within a two-gene operon and is proximal to BAS5182, which encodes fructose-1,6-bisphosphatase, class II (1).

The *B. anthracis* genome contains other examples of multiple variants of important cellular targets. Unlike most characterized bacteria, *B. anthracis* contains two distinct *murB* genes (11, 16) that encode the enzyme responsible for the cell wall biosynthesis step immediately following that of MurA. In addition, *B. anthracis* contains an apparent gene duplication of the methionyl tRNA synthetase gene *metRS* (2, 10, 11), which is rare among sequenced bacterial species, although two functional *metRS* genes have been demonstrated in some *S. pneumoniae* clinical isolates (2, 10). In earlier work, we used targeted antisense and gene replacement mutagenesis techniques to show that only one each of the *murB* and *metRS* genes contribute to essential cellular function (11). Moreover, antisense-based reduction of functional *murB2* and *metRS1* expression hypersensitized the growth of *B. anthracis* cells to specific antibiotics, thereby demonstrating that these genes are viable antibiotic development targets against this pathogen. In light of these experiments, we assessed the relative functional contributions of the two *B. anthracis murA* genes to cell wall biosynthesis and the activity of fosfomycin on *murA1* antisense modulated cells.

MATERIALS AND METHODS

Culture media, strains, and plasmids. *B. anthracis* strains were grown at 37°C in brain heart infusion (BHI; Difco, Kansas City, MO) or cationic adjusted Mueller-Hinton II broth (Becton Dickinson, Franklin Lakes, NJ), supplemented with chloramphenicol, erythromycin, or kanamycin (Sigma-Aldrich, St. Louis, MO). The *B. anthracis* plasmidless strain UM23C1-1 was obtained from Stephen H. Leppla (15). pSABA-3 was previously published (11) and is summarized below.

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DNA manipulations and analysis. Standard DNA manipulation techniques were used (18). Transformation of *E. coli* DH5 α and INV110 competent cells was conducted according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Restriction and modification enzymes were obtained from New England Biolabs (Beverly, MA). DNA sequencing services and custom synthesis of oligonucleotides were provided by Retrogen, Inc. (San Diego, CA).

Antisense library construction. The antisense screens of the *B. anthracis* *murA1* and *murA2* genes were conducted as previously described (11) and are summarized as follows: the *murA* genes were each PCR amplified from *B. anthracis* genomic DNA with primers that also encompassed about 200 bp of the 5' and 3' flanking sequences. The resulting amplicons (*murA1*, 1,542 bp; *murA2*, 1,477 bp) were fragmented by sonication to a size range of 100 to 400 bp in length. These fragments were inserted into the polylinker SmaI restriction site of the *B. anthracis* expression plasmid pBAX-2 (11), and recombinants were rescued and propagated in the *E. coli* bacterial strain DH5 α (Invitrogen). The sequences of randomly selected clones from each resulting expression library were confirmed, and the location and orientation of each insert was determined.

Antisense-induced hypersensitivity testing. Plasmid DNA from library clones was transformed into *B. anthracis* UM23C1-1 for antisense hypersensitivity screening. Twofold serial dilutions of known compounds, along with fosfomycin, were tested against *B. anthracis* antisense strains in the presence of slightly growth inhibitory concentrations of xylose (*murA1*, 40 mM; *metRS1*, 60 mM), as well as without xylose in BHI broth plus chloramphenicol at 34 μ g/ml. Each dose-response experiment was performed with four replicates at each dose, and the standard deviations were plotted. The 50% inhibitory concentration (IC₅₀) shift is defined as the ratio of the 50% growth inhibition concentration for the drug in the absence or presence of xylose. To determine the specificity of a panel of antibacterial drugs, dose-response experiments were performed in duplicate. Graphpad InStat (version 3) was used for statistical analysis. Mean IC₅₀ values of compounds for control versus treated groups were compared by using paired *t* test analysis at α level of 0.05. Two-tailed *P* values were calculated to determine the significance of the test. A *P* value of <0.05 was considered to be a significant difference in the means of treated versus control groups.

Gene replacement. Gene replacement to create *murA1* and *murA2* null mutations was carried out as previously described (9). Flanking sequences of the target gene were cloned on either side of a kanamycin resistance (Kan^r) gene in the insertion vector pSABA-3 (11), which contains both the pUC18 origin of replication and the partial pT181 rolling-circle origin of replication that requires the presence of the *repC* gene in *trans* in order for replication to occur. Also present on this plasmid are the selectable markers for erythromycin resistance (Ery^r) to monitor the plasmid backbone. When transformed into *B. anthracis* UM23C1-1, the target gene sequences flanked the Kan^r gene direct insertion of the plasmid on either side of the target gene, thereby creating duplicate flanking sequences on either side of the target gene and the Kan^r gene. A second recombination event within the duplicated flanking sequences can then excise the plasmid, either leaving behind the gene of interest or the Kan^r gene in its place. This excision event can be efficiently counterselected for by transforming plasmid insertion strains with a second autonomously replicating plasmid, pRX3-*repC* (11), which carries chloramphenicol resistance (Chl^r) and imposes the toxic effects of rolling-circle replication on the pSABA-3 plasmid backbone inserted in the genome. Thus, all Chl^r pRX3-*repC* transformants selected have excised the pSABA-3 backbone, as indicated by loss of the Ery^r marker. Chl^r Ery^s strains are evaluated for resistance to kanamycin. Kan^s colonies would result from a crossover leading to restoration of the wild-type gene, whereas Kan^r colonies result from the loss and/or replacement of the target gene with the Kan^r gene. For nonessential genes, the ratio of Kan^r to Kan^s colonies should be 1:1. These results are confirmed by PCR using primers external to the region and sequencing the resulting PCR products (data not shown).

Northern blotting. Two representative clones, 2G2 and 1A4, were analyzed by Northern blotting. Cultures (10 ml) of these two clones were grown at 37°C to a cell optical density at 600 nm of 0.1 in Mueller-Hinton-CA broth plus 34 μ g of chloramphenicol/ml. At this density, 5 ml of each culture was transferred into prewarmed flasks containing xylose at a final concentration of 133 mM. After 90 min of further incubation with shaking at 37°C, the cultures were harvested. RNA was isolated and purified by using a RiboPure bacterium kit (Ambion, Inc., Austin, TX). Total RNA was denatured at 55°C for 10 min in RNA sample loading buffer and was analyzed on a 1% agarose gel containing 2% formaldehyde. StarFire probes (Integrated DNA Technologies, San Diego, CA) to *murA1* and *metRS1* were labeled with [³²P]dATP. The blots were probed at 60°C in Rapid-Hybe buffer (GE Healthcare, Piscataway, NJ), washed with 1 \times SSC (0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate, and exposed to Kodak BioMax film for 3 days.

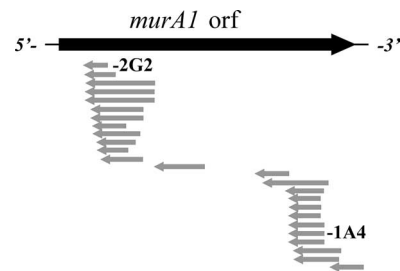


FIG. 1. Twenty-five *murA1* xylose-sensitive clones obtained from screened isolates. The bold arrow indicates the *murA1* open reading frame; the shaded arrows indicate the sizes and locations of the xylose-sensitive antisense clones. Representative clones 2G2 and 1A4 were chosen for further characterization.

RESULTS AND DISCUSSION

Targeted antisense involves an empirical selection of gene fragments that, when conditionally expressed in the antisense orientation, cause phenotypic changes that are characteristic of debilitating or null mutations of that gene. Although MurA enzyme activity is essential for viability, it is possible that antisense-based reduction in the expression of one *murA* gene might be compensated for by expression from the second *murA* gene.

To assess the contribution of each gene to viability, expression libraries of fragments from the *B. anthracis* *murA1* and *murA2* genes were prepared. Antisense clones were identified by using a method described previously (11). Sequencing of randomly selected clones from each resulting expression library showed that gene fragment inserts were represented equally in the sense or antisense orientation.

Plasmid DNA was isolated from each library and then amplified through the *E. coli* *dam/dcm* mutant strain INV110 (Invitrogen) prior to electroporation into the avirulent *B. anthracis* strain UM23C1-1 (15). Approximately 4,000 *B. anthracis* transformants were selected and cultured overnight in BHI plus 10 μ g of chloramphenicol/ml (Chl10). The resulting individual cultures were replica plated onto BHI plus Chl10 solid medium either lacking or containing 2% xylose. Transformant *B. anthracis* colonies that failed to grow on xylose-containing medium, due to the induction of gene fragment expression from pBAX-2, were selected for further study.

Primers flanking the pBAX-2 insertion site (BAXPRO and RNLX2) were used to PCR amplify the insert from each growth-sensitive transformant. The PCR products were then sequenced (Retrogen, Inc., San Diego, CA) using these primers to determine the relative position and orientation of the fragment relative to the original gene of interest. Of the 25 xylose-sensitive clones obtained, 24 of the inserts were found to be localized to the 5' and 3' thirds of the *murA1* open reading frame, with the central region relatively devoid of growth-inhibitory fragments (Fig. 1). All of the fragments were found to be expressed in the antisense orientation relative to the *murA1* gene, even though gene fragments in both orientations were equally represented in the original library. In contrast, no growth inhibitory colonies were detected after an equivalent number of transformants from the *murA2* library were screened. This suggests that only *murA1* is essential for viability

in *B. anthracis*, a result that differs from the findings for the *murA* genes of *S. pneumoniae*, where either gene alone is sufficient for growth (6).

To confirm the *B. anthracis* findings, gene replacement was used to test the phenotypic effects of *murA1* and *murA2* null mutations. In this technique, sequences flanking the *murA1* and *murA2* genes were inserted on either side of a Kan^r gene in the vector pSABA-3 (Ery^r) (11) and transformed into *B. anthracis* UM23C1-1, resulting in insertion of the plasmid into the genome. A second recombination event was selected by transformation with pRX3-*repC* (Chl^r) (11), leaving behind either the gene of interest or the precise insertion of the Kan^r gene in its place. All Chl^r pRX3-*repC* transformants lost Ery^r, indicating excision of the pSABA-3 backbone. Among 291 Chl^r pRX3-*repC* transformants in a *murA2*::pSABA-3 insertion strain, 265 were resistant to kanamycin. Four of these Kan^r Chl^r Ery^s isolates were randomly chosen for further analysis and were confirmed to lack the *murA2* gene due to its replacement with the Kan^r gene. The growth rates of these *murA2* gene replacement strains did not differ significantly from wild-type UM23C1-1, confirming that the *murA2* gene is dispensable for normal vegetative growth. In contrast, none of the 409 Chl^r Ery^s pRX3-*repC* transformant colonies of a *murA1*::pSABA-3 strain were found to be Kan^r, suggesting that only restoration of the *murA1* gene and not its replacement with the Kan^r gene is a survivable event. It should be noted that the *murA1* gene is the most distal gene in a putative three gene operon, and therefore precise replacement of this gene with the Kan^r gene should not be detrimental to the expression of the proximal genes. Therefore, both the high yield of growth-inhibitory antisense fragments obtained for *murA1* and the absence of viable bacteria upon excision of *murA1* confirmed the essentiality of this particular gene homolog.

Multiple genes for important cellular targets exist for many bacteria. Several studies have highlighted the different strategies that bacteria utilize: (i) only one gene of a set of paralogs encodes the essential function; or (ii) more than one gene encodes a protein that contributes to the essential function, and therefore multiple gene knockouts are necessary to demonstrate the essential nature of the particular function. The knockout data presented here suggest that only *murA1* is essential in *B. anthracis*, whereas other work has shown that both *murA* genes of *S. pneumoniae* encode proteins that contribute to cell wall biosynthesis (6). Similarly, we have shown that *B. anthracis*, unlike most characterized bacteria, contains two distinct *murB*-like genes (11, 16), only one of which (*murB2*) is essential for cell wall biosynthesis. In addition, of the two apparent *B. anthracis* methionyl tRNA synthetase genes (*metRS*), only *metRS1* is functional and essential (2, 10, 11). This is again different from the finding of two functional *metRS* genes in some *S. pneumoniae* clinical isolates (2, 10). Lastly, we have shown that in *B. anthracis*, of the two *dxr* gene homologs putatively involved in mevalonate-independent isoprenoid biosynthesis, only *dxr-2* encodes the essential function (12). Thus, since the contribution of specific genes to an essential function may differ for different species, it is critical that suppositions about essentiality based upon one species should not be applied to other species without empirical testing. In light of the essentiality knockout data for the *murA1* gene, we investigated whether antisense-based expression attenuation could cause

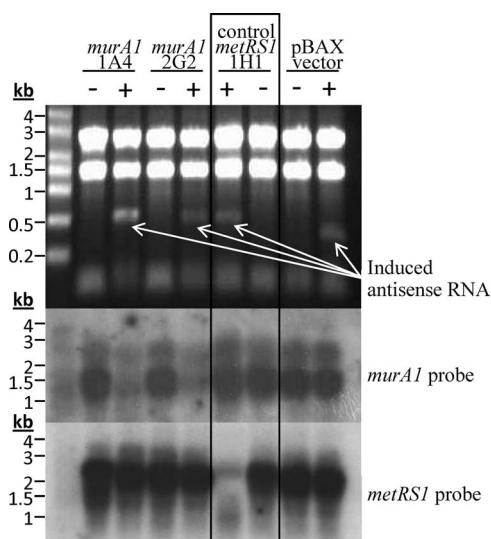


FIG. 2. Northern blot of *murA1* mRNA degradation upon induction of gene specific antisense RNA. Top panel, agarose gel; middle panel, Northern blot of agarose gel probed with *murA1* probe; bottom panel, Northern blot of agarose gel probed with *metRS1* probe.

phenotypic changes characteristic of a conditional *murA1* null mutation. Twelve sequence-confirmed *murA1* antisense clones were tested and found to exhibit growth inhibition in the presence of xylose, supporting the hypothesis that *murA1* is essential. Two representative *murA1* antisense clones, 2G2 (109 bp) and 1A4 (159 bp) (Fig. 1), and one *metRS1* (96 bp) clone (11) were analyzed by Northern blotting. Figure 2 shows (top panel, lanes 3 and 5) the induction of *murA1* antisense mRNA in response to xylose and shows (top panel, lane 6) the induction of *metRS1* antisense in the *murA1* and *metRS1* clones. It should be noted that the control vector produces RNA upon induction. Thus, the sizes of 1A4, 2G2, and 1H1 reflect the size of the insert plus the vector RNA. The data in Fig. 2 (bottom panel, lanes 3 and 5) confirms the loss of *murA1* mRNA in response to induction by xylose, whereas *metRS1*-specific mRNA is unaffected. The control *metRS1* clone demonstrates the opposite results: *murA1* mRNA is unaffected, while *metRS1* mRNA is lost upon xylose induction, presumably due to mRNA degradation.

Previous studies have shown that decreased expression of target proteins often results in hypersensitivity to compounds that specifically inhibit that protein (14). This phenomenon has been the rationale behind many drug discovery antimicrobial whole-cell screens using conditional mutants under moderately suppressing conditions to identify specific inhibitors. We anticipated that the production of *murA1* antisense would result in a concomitant reduction of MurA1 protein and hypersensitivity to fosfomycin, a specific MurA inhibitor. Strain 2G2 was chosen for fosfomycin hypersensitivity experiments, wherein the growth response was measured in the absence of xylose and the presence of increasing twofold concentrations of fosfomycin ($IC_{50} = 126 \mu\text{g/ml}$, Fig. 3). The resulting dose-response curves reflected the high fosfomycin resistance of the UM23C1-1 strain background ($MIC = >2,048 \mu\text{g/ml}$). However, in the presence of xylose, the fosfomycin dose-response curve was shifted drastically ($P = 0.0006$) to lower effective

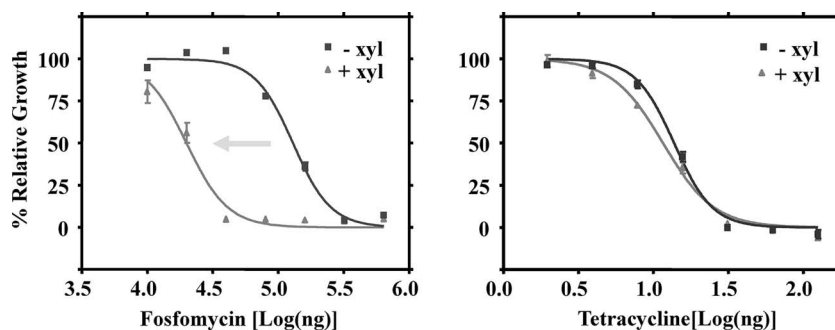


FIG. 3. Dose-response curves for AS clone 2G2 generated in the presence or absence of xylose. Left panel, relative growth versus fosfomycin concentration showing a significant shift ($P = 0.0006$); right panel, relative growth versus tetracycline concentration showing no significant shift ($P = 0.142$).

fosfomycin concentrations ($IC_{50} = 18 \mu\text{g/ml}$), indicating strong (sevenfold) hypersensitivity to the MurA-specific drug (Fig. 3). No such shift in dose response ($P = 0.142$) was observed for an unrelated antibiotic (tetracycline) in either the presence or the absence of equivalent doses of xylose (Fig. 3), nor for a number of antibiotics that target non-cell-wall-related cellular functions (Fig. 4). Figure 4 shows that the *murA1* antisense strain was hypersensitive to the β -lactam drugs piperacillin and oxacillin ($P = 0.0192$ and 0.013 , respectively), a finding similar to the *murB2* antisense strain hypersensitivity we previously reported (8, 11). This apparent synergy has also been noted as an effect of fosfomycin (22), as well as the experimental expression attenuation of other genes in the peptidoglycan biosynthesis pathway (9, 11, 17, 21). A modest yet significant degree of *murA1*-specific hypersensitivity ($P = 0.029$) was observed for the glycopeptide antibiotic vancomycin, which also affects murein transpeptidation. In comparison, when a *metRS1* antisense strain (11) was tested against the same antibiotic panel, significant hypersensitization ($P = 0.0069$) was only observed for the methionyl tRNA synthetase specific lead compound Rx100019 (8, 11).

The induction of *murA1* antisense resulted in the hypersensitization of UM23C1-1 cells to fosfomycin. Despite the hyper-

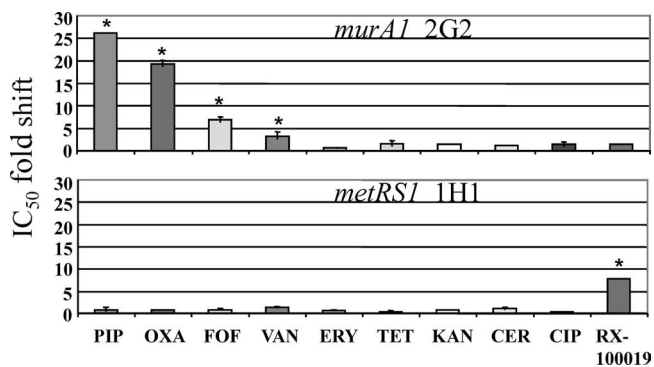


FIG. 4. Fold change in IC_{50} versus a panel of antibiotics. The fold change was determined by dividing the IC_{50} in the absence of xylose by the IC_{50} in the presence of xylose. Antibiotic panel: PIP, piperacillin; OXA, oxacillin; FOF, fosfomycin; VAN, vancomycin; ERY, erythromycin; TET, tetracycline; KAN, kanamycin; CER, cerulenin; CIP, ciprofloxacin; RX100019, MetRS1-specific investigational compound. P values were determined for treated samples versus the untreated control. *, $P < 0.05$.

sensitization of UM23C1-1 cells to fosfomycin by *murA1* antisense expression and the demonstrated lack of essential functionality of the *murA2* gene, the mechanism of fosfomycin resistance in this strain is still unclear. The *B. anthracis* Ames genome sequence (16) contains two open reading frames (BA_2042 and BA_4109) that encode proteins with strong homology to the *B. subtilis* FosB protein (3), with BA_4109 likely to be phage derived (18). The FosB protein is a Mg^{2+} -dependent cysteine thiol transferase enzyme that modifies and inactivates fosfomycin and has been demonstrated to confer fosfomycin resistance when expressed in *E. coli*. The role of these BA_2042 and BA_4109 in the fosfomycin resistance of *B. anthracis* requires additional study.

We have presented here mechanistic evidence for the inhibition of *murA1* activity in *B. anthracis*. The demonstration of reduction of *murA1* cellular activity with fosfomycin due to (i) antisense expression hypersensitization or (ii) functional synergy with β -lactam drugs (22) substantiates *murA1* as a target for the development of new and effective treatments against this important biowarfare pathogen, *B. anthracis*.

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