

Evaluation of the *metS* and *murB* Loci for Antibiotic Discovery Using Targeted Antisense RNA Expression Analysis in *Bacillus anthracis*[∇]

G. C. Kedar, Vickie Brown-Driver, Daniel R. Reyes, Mark T. Hilgers, Mark A. Stidham, Karen Joy Shaw, John Finn, and Robert J. Haselbeck*

Trius Therapeutics, San Diego, California

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The biowarfare-relevant bacterial pathogen *Bacillus anthracis* contains two paralogs each of the *metS* and *murB* genes, which encode the important antibiotic target functions methionyl-tRNA synthetase and UDP-*N*-acetylenolpyruvylglucosamine reductase, respectively. Empirical screens were conducted to detect and characterize gene fragments of each of these four genes that could cause growth reduction of *B. anthracis* when inducibly expressed from a plasmid-borne promoter. Numerous such gene fragments that were overwhelmingly in the antisense orientation were identified for the *metS1* and *murB2* alleles, while no such orientation bias was seen for the *metS2* and *murB1* alleles. Gene replacement mutagenesis was used to confirm the essentiality of the *metS1* and *murB2* alleles, and the nonessentiality of the *metS2* and *murB1* alleles, for vegetative growth. Induced transcription of RNA from *metS1* and *murB2* antisense-oriented gene fragments resulted in specific reduction of mRNA of their cognate genes. Attenuation of MetS1 enzyme expression hypersensitized *B. anthracis* cells to a MetS-specific antimicrobial compound but not to other antibiotics that affect cell wall assembly, fatty acid biosynthesis, protein translation, or DNA replication. Antisense-dependent reduction of MurB2 enzyme expression caused hypersensitivity to beta-lactam antibiotics, a synergistic response that has also been noted for the MurA-specific antibiotic fosfomycin. These experiments form the basis of mode-of-action detection assays that can be used in the discovery of novel MetS- or MurB-specific antibiotic drugs that are effective against *B. anthracis* or other gram-positive bacterial pathogens.

Bacillus anthracis is the etiological agent of the infectious disease anthrax. The inhalational form of the disease is rare in humans; however, the demonstrated efficacy of anthrax as a biological weapon is a new health threat that could affect large populations. Fortunately, *B. anthracis* is sensitive to the fluoroquinolone and tetracycline classes of antibiotics (15, 35), and timely prophylactic use of these drugs has been recommended for reduction of the large-scale morbidity and mortality resulting from an anthrax attack on an urban population center (4, 9, 15, 67). However, this treatment option may prove ineffective in the face of a bioweaponized *B. anthracis* strain that is resistant to these drugs. *B. anthracis* strains have been shown to exhibit significant rates of spontaneous resistance to ciprofloxacin and doxycycline (3, 9, 26), while high levels of intrinsic resistance to other generally useful antibiotics, such as trimethoprim, further limit alternative methods of treatment (1, 5, 6, 46). Moreover, Soviet scientists are now known to have purposely engineered multiple drug resistance mechanisms into *B. anthracis* strains intended for bioweapon production (50, 60).

One aspect of the general antibacterial resistance problem is that, despite decades of research and development efforts, the number of distinct antibacterial classes and the cellular functions that they inhibit remain limited. Most antibacterials in use today target cell wall biosynthesis by inhibiting the action of penicillin binding proteins (beta-lactams and glycopeptides),

protein translation by inhibiting various functions of the ribosome (macrolides, tetracyclines, and aminoglycosides), or DNA replication by inhibiting the enzymes DNA gyrase and topoisomerase I (fluoroquinolones). Extensive use of these antibiotics over the past decades has provided the selective pressure for the development of resistance mechanisms in these pathogenic bacteria. The discovery of novel antibacterial agents that inhibit other unexploited essential cellular functions sidesteps these common resistance mechanisms, providing effective alternatives to the current arsenal of clinically useful antibacterials.

To provide new treatment alternatives for anthrax and other bacterial diseases, we are using target-based approaches to discover antibiotics that act at novel or unexploited functions within bacterial cells. Two targets of interest are methionyl tRNA synthetase (MetS, sometimes abbreviated MetRS) and UDP-*N*-acetylenolpyruvylglucosamine reductase (MurB). MetS is 1 of 20 enzymes that charge amino acids to their cognate tRNAs. MetS provides a logical and potent alternative inhibition point in protein synthesis, as the resulting charged methionyl-tRNA is used both for translation initiation and elongation by the bacterial ribosome. Several classes of MetS inhibitors with antibacterial activity have been reported (12, 19, 25, 30), including one that is in preclinical development (12, 45). The MurB enzyme, UDP-*N*-acetylenolpyruvylglucosamine reductase, catalyzes an early and crucial step in the cell wall biosynthesis pathway. The subsequent steps in the pathway are entirely dependent on the MurB product UDP-MurNAc for completion and delivery of peptidoglycan monomer units to actively growing regions of the bacterial cell wall. Due to its importance in peptidoglycan biosynthesis, the MurB enzyme

* Corresponding author. Mailing address: Trius Therapeutics Inc., 6310 Nancy Ridge Dr., Suite 105, San Diego, CA 92121. Phone: (858) 452-0370, ext. 107. Fax: (858) 452-0412. E-mail: rhaselbeck@triusrx.com.

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has been the subject of a number of target-based drug design efforts (8, 18, 21, 36, 39, 69).

Identification of novel antibacterial agents that act by inhibiting either MetS or MurB is a substantial challenge. Compounds that are found to inhibit these enzymes in cell-free biochemical assays may later prove to lack significant antimicrobial activity. Conversely, the detection of MetS- or MurB-specific antimicrobial compounds from high-throughput compound library screens could prove overly cumbersome with currently available methods for mode-of-action (MOA) determination. Recently, effective and facile antisense-based MOA detection methods have been described in *Staphylococcus aureus* (20, 32, 34). High levels of RNA expression from antisense-oriented target gene fragments can cause bacterial growth inhibition if the target gene is required for growth, an attribute that has been used to survey genes for essentiality in *S. aureus* (20, 32, 34). If antisense RNA expression is modulated, cellular growth becomes hypersensitized to the further addition of antimicrobial compounds that act specifically on that target cell function (20, 33). Such an assay has led to the discovery of promising antibacterials from a large natural product compound library that specifically act on the *S. aureus* fatty acid biosynthesis enzymes FabF and FabH (20, 47, 66, 71).

Here we show the effectiveness of this antisense-based approach as an essential gene detection tool and antimicrobial MOA assay in *B. anthracis*. Although there are two paralogs of each of the *metS* and *murB* genes in *B. anthracis*, we detected significant numbers of growth-inhibitory antisense fragments to only those paralogs that we also showed to be essential for growth by gene replacement mutagenesis. The growth reduction caused by expression of these antisense fragments correlates with specific reduction of mRNA levels of the target gene. *B. anthracis* strains containing verified antisense fragments of *metS* or *murB* were specifically hypersensitive to the addition of antimicrobial compounds that target methionyl-tRNA synthetase or cell wall biosynthesis, respectively. We describe how these MOA assays can be used to discover or to verify MetS-specific or MurB-specific antimicrobial compounds.

MATERIALS AND METHODS

Culture media and growth conditions. *B. anthracis* strains were grown at 37°C in brain heart infusion (BHI; Difco, Kansas City, MO), LB medium (BD Biosciences, San Jose, CA), or cationic-adjusted Mueller-Hinton II broth (MH; Becton Dickinson, Franklin Lakes, NJ), supplemented with chloramphenicol (Cm), erythromycin (Em), or kanamycin (Km) (all obtained from Sigma-Aldrich, St. Louis, MO). Antibiotic concentrations routinely used for culture of *B. anthracis* were 5 µg ml⁻¹ for Em (Em5), 50 µg ml⁻¹ for Km (Km50), and 10 µg ml⁻¹ for Cm in solid media (Cm10) or 34 µg ml⁻¹ (Cm34) in liquid media. *Escherichia coli* strains used as hosts for cloning were cultured in LB medium (BD Biosciences, San Jose, CA) supplemented with carbenicillin (MP Biomedicals, Solon, OH) at a final concentration of 100 µg ml⁻¹ (Cb100). Agar plates were made by adding 1.5% agar (Difco, Kansas City, MO) to liquid BHI or LB media. Xylose (Sigma-Aldrich, St. Louis, MO) was added to BHI agar plates at a final concentration of 2% for screens of antisense-sensitive clones or to empirically determine final concentrations in liquid media.

Strains and plasmids. *E. coli* strains DH5α and INV110 were obtained as prepared CaCl₂-competent cells from Invitrogen, Inc. (Carlsbad, CA). *B. anthracis* plasmid-less strain UM23C1-1 (52) was obtained from Stephen H. Leppla. Plasmid pSWEET-*bgaB* (7) was obtained from the laboratory of Eric Brown. Plasmids pSA3528 and pSA7592 (68) were obtained from Serban Iordanescu. Plasmid pDL276 (17) was obtained from Gary Dunny. Plasmid pVA838 (40) was ob-

tained from the American Type Culture Collection, Manassas, VA. Plasmid pUC19 (51) was obtained from Invitrogen, Inc. (Carlsbad, CA).

DNA manipulations and analysis. Standard DNA manipulation techniques were used as described previously (58). Restriction and modification enzymes were obtained from New England Biolabs (Beverly, MA). DNA sequencing services, as well as custom synthesis of oligonucleotides used in this study, were provided by Retrogen Inc. (San Diego, CA). *B. anthracis* genomic DNA was isolated and purified with the use of a commercially available kit (GENTRA, Inc., Minneapolis, MN). Plasmid DNA was purified with the use of Miniprep kits from QIAGEN GmbH (Hilden, Germany) and with Wizard Midiprep kits from Promega Corporation (Madison, WI). PCR products were amplified with the use of either an AccuPrime *Taq* DNA polymerase system kit or with a high-fidelity Platinum *Pfx* DNA polymerase kit (Invitrogen, Inc., Carlsbad, CA). DNA fragments were prepared for molecular biology manipulations with QIAquick DNA purification kits (QIAGEN GmbH, Hilden, Germany). Transformation of *E. coli* DH5α and INV110 competent cells was conducted according to the manufacturer's instructions (Invitrogen, Carlsbad, CA).

Construction and characterization of plasmid pBAX-2, a xylose-inducible expression vector. Plasmid pLEX5B (16) was digested with HindIII and XmnI to delete the existing polylinker. After the DNA was blunt ended with Klenow fragment of DNA polymerase, it was recircularized by ligation, thereby restoring a HindIII site at the junction. The resulting plasmid, pRX3-30, was then linearized with HindIII and mixed and ligated to HindIII-linearized plasmid pC194 (27). One recombinant plasmid, in which the Am^r gene of pLEX5B and the chloramphenicol resistance (Cm^r) gene of pC194 were in the same orientation, was denoted pRX3-31. This plasmid was then subjected to partial digestion with HindIII and then complete digestion with XhoI, followed by end polishing with Klenow fragment and ligation to recircularize. This manipulation destroyed one HindIII site while leaving the other HindIII site proximal to the 3' end of the ampicillin resistance gene intact. The resulting plasmid, pRX3-32, was then digested with HindIII to accommodate a double-stranded polylinker made by annealing the oligonucleotides POLYCASS FOR and POLYCASS REV (Table 1) to create pRX3-33 (Fig. 1). The *Bacillus subtilis* *xylR* gene and *xylA* promoter were PCR amplified from pSWEET-*bgaB* (7) using Platinum *Pfx* DNA polymerase and the NheI-tailed oligonucleotides XAMP-FOR and XAMP-REV (Table 1). Care was taken to omit potential ribosome binding sites and start codons in the transcribed region downstream of the promoter, as well as a *cis*-acting CRE site within the *xylA* coding region (38). The resulting 1.5-kbp DNA fragment was then inserted in the NheI site of plasmid pRX3-33 in the orientation shown to create plasmid pBAX-2 (Fig. 1).

Library construction. The sequence information of *B. anthracis* *metS1* (BA0036), *metS2* (BA5278), *murB1* (BA4048), and *murB2* (BA5315) genes was derived from the GenBank entry for the complete genome sequence of *B. anthracis* strain Ames (gi/30260185) (54). This information was used to design oligonucleotide primer pairs (METS1-FOR/REV, METS2-FOR/REV, MURB1-FOR/REV, and MURB2-FOR/REV) (Table 1) that were used to amplify the open reading frame of each gene including about 200 bp of 5' and 3' flanking sequence, using Platinum *Pfx* DNA polymerase and *B. anthracis* UM23C1-1 genomic DNA as a template. Resulting fragments in the size range of 100 to 600 kbp were then blunt-end polished with T4 DNA polymerase and Klenow fragment of DNA polymerase, dephosphorylated by treatment with calf intestinal phosphatase, and then ligated with SmaI-digested plasmid pBAX-2 (relative molar ratio, 5:1) using T4 DNA ligase at 16°C for 18 h. The DNA was then subjected to a second SmaI digest to eliminate reclosed vector, as there were no SmaI sites apparent within the amplified genomic regions (54). After this treatment, the DNA was transformed into *E. coli* DH5α maximum competent cells (Invitrogen, Carlsbad, CA) and then plated on LB agar with Cb100. From the resulting colonies, 48 were randomly selected for PCR amplification with oligonucleotides RX3-2 and BAXPRO (Table 1; Fig. 1) to gauge the size and orientation of fragment inserts within the pBAX-2 SmaI site. Libraries that yielded at least 10,000 transformant colonies with an insertion frequency greater than 80% were deemed suitable for screening. Transformant colonies were scraped from the agar surface and pooled and then plasmid DNA was purified using a Wizard Midiprep kit (Promega, Madison, WI). Library DNA was then amplified by transformation in and purification from the *dam/dcm* *E. coli* strain INV110, a necessary step to ensure maximal electroporation efficiency of library DNA in *B. anthracis* (41).

Screen for clones imparting xylose-inducible growth sensitivity. *B. anthracis* UM23C1-1 cells (52) were made electroporation competent using a previously described protocol (49). Electroporation of library plasmid DNA into UM23C1-1 cells was conducted as follows: 100 µl of electrocompetent UM23C1-1 was mixed with 1 µg of library DNA, placed in an ice-cold 2-mm electroporation cuvette, and then subjected to one pulse at 1.9 kV, 25 µF, 200 Ω. One ml of SOC medium

TABLE 1. Oligonucleotides used for plasmid construction, genetic characterization, and measurements of transcriptional levels^a

Primer use and name	Oligonucleotide sequence (5' to 3')
Construction and sequencing primers	
XAMP-FOR.....	..ATAGCTAGCATGATAAGCTGTCAAACATGAGAAT
XAMP-REV.....	..ATAGCTAGCGTAAGTAAGTTGCACATTAGTTTGTGTTG
RX3-1.....	..AAATCGACACTGAATTTGCTC
RX3-2.....	..CACTTCTGAGTTCGGCATGG
BAXPRO.....	..TATGAGATTTAGTACATAGCGAATCTTACC
METS1-FOR.....	..TCGGGTAAATAGTCAGCAGGA
METS1-REV.....	..TCGTTGCTTTCATCAAACCA
METS2-FOR.....	..TCGGTAGCTGAGAAGAAGCA
METS2-REV.....	..CCTCGGATTATTCGGTGTG
MURB1-FOR.....	..AAATGACCGTTCTGTTGTCTG
MURB1-REV.....	..ATTGCTAAGCGGAGATCGAA
MURB2-FOR.....	..GACATGTCAAGTTTAGTAGCCCATT
MURB2-REV.....	..TCCAGAGTGAATTCGCAAGTTT
PUCFOR.....	..ATAATAGAATTCGCATTAATGAATCGGCCAAC
PUCREV.....	..ATAATAGAATTTCTTTTCGGGAAATGTGC
EMSAFOR.....	..ATAATACAATTTGCCGTTGTAAAAATCGGAGAA
EMSAREV.....	..ATAGAATTCAGACTCTGTCGACTATTATGGATCCTTATTTCCCTCCCGTTAAATAATAGA
KANFOR.....	..ATAATAGGCGCGCCCGAACCATTTGAGGTGATAGGTAAG
KANREV.....	..ATAATAGCGCGCCTCTAGGTACTAAAACAATTCATCCAG
POLYCASS FOR.....	..AGCTGGCTAGCTTGCCAGTGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTTCGACCTGCAGCCA
POLYCASS REV.....	..AGCTTGCTGCAGGTGACTCTAGAGGATCCCGGGTACCGAGCTCGAATTCAGTGGCAAGCTAGCC
SABA-1.....	..GTTTCAAAATGGGTCAATCG
SABA-2.....	..TTTTTCGTCCATGTGAACTG
KANFOROUT.....	..CCTATCACCTCAAATGGTTCG
KANREVOUT.....	..TTTTTGACTTACTGGGGATCAAG
RT-PCR primers	
RX3V1-F.....	..CCATGCCGAAGTTCAGAAAGTGA
RX3V1-R.....	..TGCTGGCAGTTCCTACTCT
RX3V1-P.....	..FAM-ACGCCGTAGCGCGGATGGTAGTG-TAMRA
MS1-F.....	..TGAGACATTTCTATACGGAGCATCAA
MS1-R.....	..TCTACATCATGACCACATATCTGGACTT
MS1-P.....	..FAM-CCAATTTATGGAAGGTGACAAAGTAGTTGGAGGA-TAMRA
MS2-F.....	..CCTGGAGATATTTTAGCACGTTATTACC
MS2-R.....	..GCAATTTGGTGTTCATTGCA
MS2-P.....	..FAM-CAAAGGTGAGAAATGTGTGTATGTTTCGGG-TAMRA
MB1-F.....	..TGAGCAATTTAGTAAATGAGCTTATACAA
MB1-R.....	..TATTTTCATAGTGGTATAACGTGCTAACG
MB1-P.....	..FAM-CAAATGTTGGTTCGCGTGTAGTGGATGAA-TAMRA
MB2-F.....	..GCCTGAAGGTGATGTGAAACAAG
MB2-R.....	..AATTTGTGCGGCAACGA
MB2-P.....	..FAM-CGATATTTAAAGTTGGTGGAAAAGCAGATGTG-TAMRA
BA16s-F.....	..TTCGGGAGCAGAGTG
BA16s-R.....	..AACATCTCACGACACGAG
BA16s-P.....	..FAM-CAGGTGGTGCATGGTTGTC-TAMRA

^a Reporter (6-carboxyfluorescein [FAM]) and quencher (6-carboxytetramethylrhodamine [TAMRA]) dyes are indicated on 5' and 3' ends of probes used for real-time RT-PCR analyses.

(Invitrogen, Inc., Carlsbad, CA) supplemented with 10% (wt/vol) sucrose was then added to the cells and then the volume was transferred to a 15-ml Falcon tube and incubated at 37°C for 90 min with gentle shaking (100 rpm). The cells were then spread onto solid BHI Cm10 medium to obtain well-separated colonies after overnight incubation at 37°C.

Over 2,000 transformant colonies from each library were inoculated and arrayed into wells of 96-well plates (Costar no. 3598; Corning, Midland, MI) containing 100 μ l of BHI Cm34 broth. After incubation for 18 h at 37°C, the resulting liquid cultures were replica plated with hand-held pin tools onto LB Cm10 solid medium in OMNI plates (Nunc 242811; Nalge Nunc International, Rochester, NY), either with or without xylose added to a 2% final concentration. These replica plates were incubated at 37°C overnight, and relative growth of individual strains on the two media was compared in order to identify strains that either failed to grow or showed visible reduction in growth only in the presence of xylose. Strains that met this criterion were subjected to a second more-quantitative replica plating experiment to confirm the observed xylose sensitivity phenotype. Overnight growth of each strain was subjected to four 10-fold serial dilutions in a 96-well culture plate. This was replica plated on solid media with and without 2% xylose. Resulting growth was scored based upon a visible dif-

ference between the xylose and nonxylose platings, e.g., a score of 4 represented lack of growth on all four dilutions in the presence of xylose compared with growth without xylose. Strong xylose sensitivity phenotypes exhibited scores of 4 or 3 (lack of growth on all but the least-dilute replica plating), while weaker xylose sensitivities exhibited scores of 2 or less. Strains with xylose sensitivity scores of 0 or 1 (i.e., those with xylose growth comparable to nonxylose growth in all or nearly all dilutions) were judged to have failed reconfirmation and were not considered further.

Xylose-sensitive clones with scores of 2 or higher were further characterized by establishing a dose-response curve for xylose-dependent growth inhibition in liquid medium. Individual cultures were grown to an optical density at 600 nm (OD₆₀₀) of 0.2 in BHI Cm34 and then diluted 1,000-fold into eight wells containing BHI Cm10 broth with xylose included at 150, 100, 75, 50, 25, 12.5, 6.25, and 0 mM final concentrations. Dimethyl sulfoxide was also added to a 2% final concentration to mimic conditions for later antibiotic addition experiments. Relative growth levels were measured by optical absorbance at OD₆₀₀ after 15 h of growth at 37°C and plotted against the xylose concentration. Resulting xylose dose-response curves were used to determine xylose concentrations that caused a 50% reduction in growth (xyl₅₀).

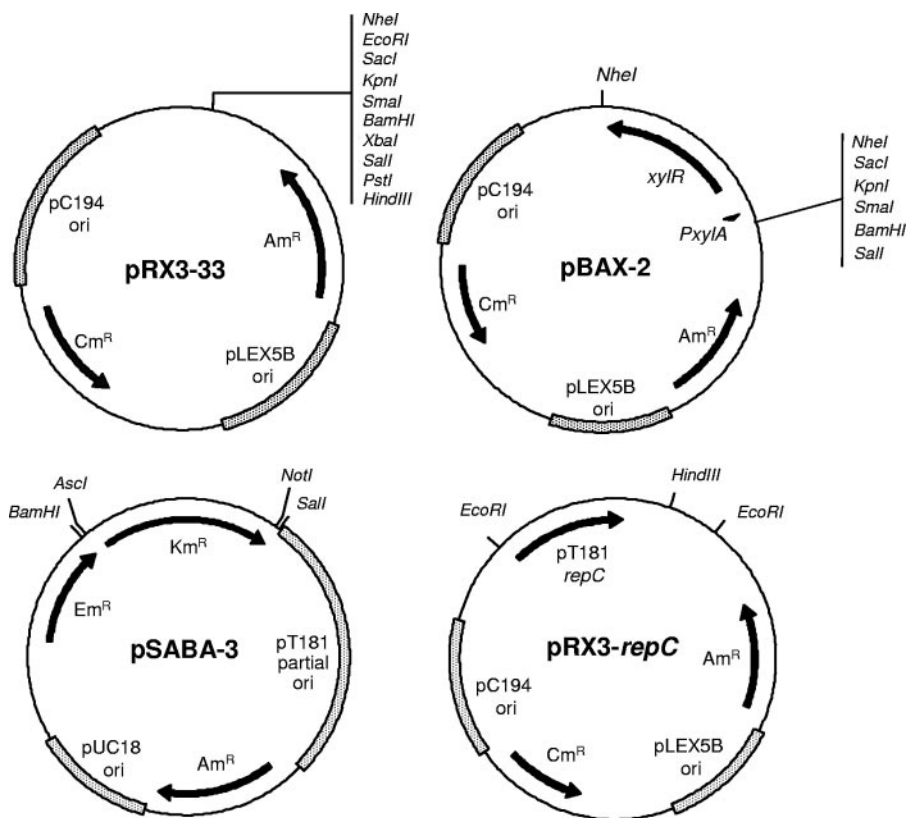


FIG. 1. Plasmids constructed for the study. Plasmid pRX3-33, a *B. anthracis*/*E. coli* shuttle vector, was used as a basis for construction of the xylose-responsive expression vector pBAX-2 and the pT181 *repC* expression vector pRX3-*repC*. The insertion vector pSABA-3 was engineered to facilitate replacement of a *B. anthracis* gene of interest with a kanamycin resistance gene.

Identification of sequence and orientation of gene fragment inserts. Concurrent with xyl_{50} experiments, the sequence of the gene inserts of each confirmed growth-inhibitory clone was determined. Oligonucleotide primers BAXPRO and RX3-2 (Table 1; Fig. 1) and Accuprime *Taq* DNA polymerase were used to amplify insert regions from colony growth of xylose-sensitive strains. Amplification products were purified using QIAquick 96-well PCR purification kits and were then subjected to DNA sequencing using the primer BAXPRO. Resulting sequence data were analyzed with the use of Sequencher (Gene Codes Corp., Ann Arbor, MI) and Clone Manager 7 (Scientific and Educational Software, Cary, NC) software.

Gene replacement mutagenesis. The insertion vector pSABA-3 was constructed as follows: a 2.5-kbp fragment from pUC18 (43) (Invitrogen, Inc., Carlsbad, CA) was PCR amplified with the EcoRI-tailed oligonucleotides PUCFOR and PUCREV (Table 1) and then digested with EcoRI. A 1.3-kbp fragment containing a gram-positive *Em^r* gene was then PCR amplified from plasmid pVA838 (40) with the MfeI-tailed EMSAFOR and the EcoRI-BglII-SalI-BamHI-tailed EMSAREV (Table 1) followed by digestion with MfeI and EcoRI. The two fragments were then mixed and ligated together to form plasmid pSABA-1A. Next, a 1.7-kbp EcoRI-BamHI DNA fragment which contains an incomplete pT181 origin of replication was excised from pSA3528 (68) and ligated into the EcoRI and BglII sites of pSABA-1A to form pSABA-2. Next, a 1.3-kbp DNA fragment containing a *Km^r* gene was PCR amplified from plasmid pDL276 (17) with the BamHI-AscI-tailed oligonucleotide KANFOR and the NotI-SalI-tailed oligonucleotide KANREV (Table 1). The resulting fragment was digested with BamHI and SalI and then inserted in the corresponding sites on pSABA-2 to create the plasmid pSABA-3 (Fig. 1).

Gene replacement insertion (GRI) plasmids for each of the *B. anthracis* *murB1*, *murB2*, *metS1*, and *metS2* genes were constructed as follows: about 1 kbp of the 5' and 3' flanking sequences of a gene of interest were PCR amplified with primers that were tailed with BamHI and AscI (5') and NotI and SalI (3') (Table 2). These amplification products were then digested with the corresponding restriction enzymes and inserted in the appropriate positions on either side of the *Km^r* gene of pSABA-3. The DNA sequences of these inserts were

confirmed using primers SABA-1, SABA-2, KANFOROUT, and KANREVOUT (Table 1).

B. anthracis UM23C1-1 strains containing GRI plasmid insertions were prepared as follows: 10 μ g of each plasmid suspended in 5 μ l of sterile water was electroporated in 100 μ l of competent cells as described above. After outgrowth for 90 min in SOC plus 10% maltose, the entire volume was plated on bioassay-size (22.5-cm²) plates containing LB agar with Km50 and Em5. Transformant colonies generally arose after 24 to 48 h of incubation at 37°C. Four colonies representing each of the four GRI plasmid transformations were then chosen for genomic mapping to confirm proper insertion of the GRI plasmids, either immediately upstream or downstream of the targeted gene of interest. Genomic DNA was prepared from each strain, which was then used as a template for PCR amplifications from within the inserted *Km^r* gene to regions either upstream or downstream of the GRI flanking sequences (Table 2). Resulting amplification products of approximately 1.5 kbp in size confirmed insertion of the GRI plasmid either upstream or downstream of the gene of interest. GRI strains were thus confirmed to contain both a gene replacement null mutant allele and a wild-type allele of the gene of interest, separated by a genomic insertion of the pSABA-3 plasmid backbone.

Confirmed GRI strains for each gene of interest were then subjected to electroporation with pRX3-*repC*, a plasmid constructed by inserting an overexpressed allele of the pT181 *repC* gene derived from plasmid pSA7592 (28, 68) into the multiple cloning site of the *B. anthracis*/*E. coli* shuttle vector pRX3-33 (Fig. 1). The resulting outgrowths from each transformation were then split into two equal-volume aliquots. One aliquot was spread on solid LB medium containing Cm10, while the other was spread on solid LB medium containing both Cm10 and Km50. After incubating for 18 h at 37°C, the number of resulting colonies on both platings was determined as a measure of gene essentiality.

RNA analysis. Xylose-sensitive *B. anthracis* strains shown to contain antisense-oriented gene fragments were characterized by real-time reverse transcription-PCR (RT-PCR) experiments to determine the effect of antisense expression on specific mRNA levels. *B. anthracis* cultures containing antisense fragment clones or the control pBAX-2 vector were grown to a cell density of 0.1 OD₆₀₀ in MH

TABLE 2. Oligonucleotides used for construction of GRI plasmids^a

Gene name	Locus no.	Primer name	Primer sequence
<i>murB1</i>	BA4048	MB1-1	AATAATGGATCCTACAATCGTTCCAAAAGCAG
		MB1-2	AATAATGGCGCGCCAAACAATCTCCCCATACTC
		MB1-3	AATAATGCGGCCGCGAGATAACGGTTATTCGTTCCG
		MB1-4	AATAATGTCGACTCATCAATGAACCATTCTTG
		MB1-5	CCGGCTCTTGCTTTAATAAG
		MB1-6	TTCATTTAAGAATATCGCAGTAAAC
<i>murB2</i>	BA5315	MB2-1	AATAATGGATCCACGATACATCTGTGAAGACATTAC
		MB2-2	AATAATGGCGCGCCTTTACGATTCTCTATTTCTATATTC
		MB2-3	AATAATGCGGCCGCGAAGATAAAGAATAACATATACGTATGAAG
		MB2-4	AATAATGTCGACCTTACGAAATTAATCTAAACCCTC
		MB2-5	GCTACAGAAGAAGTGTAGCAAG
		MB2-6	GCTGATTTACCAAACCTTTGTACC
<i>metS1</i>	BA0036	MS1-1	AATAATGGATCCCCTGGTGGTGTATTTGTATTTAGTAG
		MS1-2	AATAATGGCGCGCCTTGAACCTCCCGAATAACC
		MS1-3	AATAATGCGGCCGCGAAGTAATTAAGACAAGAAAAGAGATG
		MS1-4	AATAATGTCGACCCTTATTGTAACATCTACTCATTTCAATC
		MS1-5	CGATGCAAGAAGTATTAGGAAATAG
		MS1-6	ATAGAATGTCCAAAACCTTTGTCTTC
<i>metS2</i>	BA5278	MS2-1	AATAATGGATCCGATGCTATAAGCAACATCATCTTC
		MS2-2	AATAATGGCGCGCCACCTTATTTGCGCAATTACTCAC
		MS2-3	AATAATGCGGCCGCGAGAAAAAGACCTTTTCAAATGC
		MS2-4	AATAATGTCGACGTA AAAACCTACTACAATCTCATACCC
		MS2-5	CAAGTGTGTAAATAATTTCTCCTAGC
		MS2-6	AACAATACTAGATAAACATCAATTTCC

^a Sets of six oligonucleotides were designed for each gene. Oligonucleotides 1 and 2 included the restriction sites BamHI and AscI, respectively, within 5' tails and were used to amplify the 5' flanking sequence of a gene of interest. Similarly, oligonucleotides 3 and 4 included 5' tails with NotI and SalI restriction sites, respectively, and were used to amplify the 3' flanking sequence of a gene of interest. Oligonucleotides 5 and 6 were designed to prime either upstream or downstream of genomic sequences represented by oligonucleotides 1 to 4. Oligonucleotide 5, in conjunction with KANFOROUT (Table 1), was used to amplify across the junction between the inserted Km^r gene and a region just upstream of the defined plasmid insertion site. Likewise, oligonucleotide 6, in conjunction with KANREVOU (Table 1), was used to confirm the junction between the inserted Km^r gene and downstream genomic sequences. The gene names are shown correlated with the locus numbers assigned to each gene during annotation of the *B. anthracis* genome sequence (54).

broth supplemented with Cm34. The cultures were diluted 20-fold into a final volume of 25 ml prewarmed MH Cm34 and then grown with shaking at 37°C for 4 h until the culture reached 0.1 OD₆₀₀. At this time, 4 ml of each sample was collected for an initial time point by centrifugation, while 10 ml of culture was transferred to a fresh prewarmed flask with xylose at a final concentration of 133 mM (2%, wt/vol). After 90 min of further incubation with shaking at 37°C, 4 ml of each culture was harvested. RNA from the initial time point and after growth in xylose was isolated and purified using the RiboPure bacteria kit (Ambion Inc., Austin, TX). Quantification and purity of resulting RNA were determined by measuring the absorbance at 260 nm/280 nm.

Real-time RT-PCR nucleotide primer/probe sets (Eurogentec, San Diego, CA) were designed to prime within the mRNA of *metS1*, *metS2*, *murB1*, and *murB2* (Table 1), upstream of sequence corresponding to antisense fragment clones. Primer/probe sets were also designed for detection of *B. anthracis* 16S rRNA as well as mRNA corresponding to the pBAX-2 plasmid immediately downstream of the xylose-responsive promoter and polylinker. One-step RT-PCR (Applied Biosystems, Foster City, CA) was carried out on 10 ng total RNA per reaction mixture (0.01 ng total RNA for 16S detection). Resulting data were analyzed by the comparative C_T method (User bulletin 2, ABI Prism 7700). Total RNA loadings were normalized to 16S rRNA values.

Antisense-based mode of action assays. The 50% inhibitory concentration (IC₅₀) of several antibacterial agents was determined for antisense strains in the presence or absence of moderate growth-inhibitory xylose concentrations. Antisense strains were cultured in flasks containing BHI Cm34 broth at 37°C to 0.2 OD₆₀₀. The cultures were then diluted 125× in BHI Cm34 either with or without xylose at the predetermined xyl₅₀ concentration for each antisense strain. Forty-five μl of the diluted cultures was then dispensed into 384-well plates containing 5 μl quadruplicate seven-point antibacterial dilution arrays, starting at a concentration equivalent to 10 times the MIC against *B. anthracis* UM23C1-1. After incubation for 15 h at 37°C, the OD₆₀₀ was measured in a TECAN Genios plate reader (Mannedorf/Zurich, Switzerland). Quadruplicate "plus-drug" values were averaged and normalized to the corresponding values from the no-drug control wells. Resulting dose-response data were plotted using GraphPad PRIZM soft-

ware (GraphPad Software Inc., San Diego, CA). The effect of xylose induction of the antisense RNA on the antibacterial dose response was calculated by dividing normalized IC₅₀ values without xylose by the IC₅₀ values in the presence of xylose. The antibacterials tested included cloxacillin, oxacillin, dicloxacillin, fosfomicin, vancomycin, clarithromycin, erythromycin, tetracycline, gentamicin, kanamycin, irgasan, cerulenin, rifampin, ciprofloxacin, and novobiocin. Also included was compound Rx-000019, a MetS-specific antimicrobial compound with significant activity against *B. anthracis* UM23C1-1 (MIC, 2 μg ml⁻¹) (11, 19).

RESULTS

Functional screen for antisense RNA species that affect growth of *B. anthracis*. A xylose-responsive expression plasmid pBAX-2 was developed to enable conditional expression of *metS* and *murB* gene fragments in *B. anthracis*. To gauge the function of the *B. subtilis* *xylR/A* xylose-inducible expression system in *B. anthracis*, we compared RNA levels expressed from the *xylA* promoter in the presence and absence of xylose, as measured by real-time RT-PCR (see Materials and Methods). After 90 min of induction, pBAX-2-derived RNA levels were fivefold higher than in uninduced cells or in cells containing pRX3-33. Expression levels in response to xylose were not significantly affected by the presence of 0.2% glucose, which is a sufficient concentration to cause catabolite repression of the naturally occurring *xylA* regulatory system in *B. subtilis* (7, 38).

The pBAX-2 expression vector was used to construct gene fragment libraries of each of the genes of interest: *metS1*,

TABLE 3. Number and orientation of growth-inhibitory gene fragments

Gene name	Locus no.	Total CFU analyzed	Xylose sensitive	% Total CFU	Sense	Antisense
<i>metS1</i>	BA0036	2,208	40	1.8	1	39
<i>metS2</i>	BA5278	2,400	17	0.7	10	7
<i>murB1</i>	BA4048	2,116	0	0.0	0	0
<i>murB2</i>	BA5315	2,024	26	1.3	0	26

metS2, *murB1*, and *murB2*. DNA sequencing of amplified inserts showed that they represented the corresponding gene of interest, had an average length of 200 bp, and were equally likely to be in either the sense or antisense orientation relative to the xylose-responsive promoter. For each library, about 2,000 transformants in *B. anthracis* strain UM23C1-1 were screened for xylose-dependent growth sensitivity. After 18 h of growth at 37°C, between 0.7 and 1.8% of the colonies representing the *metS1*, *metS2*, and *murB2* libraries (Table 3) showed significantly debilitated growth on agar plates only in the presence of xylose, while no such growth inhibition was observed after screening similar numbers of *murB1* library transformants.

DNA sequencing was used to determine the identity and orientation of gene inserts recovered from xylose-sensitive *metS1*, *metS2*, and *murB2* library transformant strains (summarized in Table 3). Of the 40 growth-inhibitory gene fragments identified from the *metS1* library, 39 were in the antisense orientation relative to the xylose-inducible promoter of pBAX-2 and to the sequence of the endogenous *metS1* gene. Most of the antisense-oriented fragments were found within the 3'-two-thirds of the coding region, while the one sense-oriented fragment that was detected appeared capable of expressing a truncated N-terminal fragment of the MetS1 protein. From the *murB2* library, 26 growth-inhibitory fragments were identified, all of which were in the antisense orientation. These antisense fragments clustered in two groups, either in the 5'-half of the coding region or near the 3' end of the coding region, separated by a 210-bp region that was apparently devoid of antisense fragment representation. Like *metS1* and *murB2*, a significant number of growth-inhibitory fragments were identified for the *metS2* gene. However, these inserts were equally likely to be in the sense or in the antisense orientation relative to the pBAX-2 promoter and to the gene of interest. Although similar numbers of *murB1* library transformants were screened, none was found that caused xylose-dependent growth inhibition.

Gene replacement mutagenesis experiments confirm the essentiality of *metS* and *murB* paralogs. The antisense orientation bias of the *metS1* and *murB2* gene fragment screens was reminiscent of the results of antisense-based essential gene screens in *S. aureus* (20). We therefore sought to determine the essentiality of each gene paralog by testing the survivability of mutagenic exchange of each coding region for a selectable drug marker gene. For this purpose, we applied a two-plasmid counterselection approach originally designed for use in *Staphylococcus aureus* (68). Since these plasmids contained tetracycline resistance elements that have been discouraged for direct experimental use in *B. anthracis* (2), we reconfigured the es-

TABLE 4. Determination of essentiality of the *metS* and *murB* paralogs by gene replacement mutagenesis^a

Gene name	Locus no.	Cm ^r CFU	Km ^r Cm ^r CFU	Km ^r /Cm ^r	Essential?
<i>metS1</i>	BA0036	89	0	0.00	Yes
<i>metS2</i>	BA5278	112	57	0.51	No
<i>murB1</i>	BA4048	184	72	0.39	No
<i>murB2</i>	BA5315	160	0	0.00	Yes

^a GRI strains representing each gene were transformed with pRX3-*repC*, and the resulting outgrowth was split into two aliquots. One was spread on solid medium containing Cm10, while the other was spread on solid medium containing both Km50 and Cm10. Significant numbers of colonies arising in the presence of Cm indicate the relative efficiency of the transformation, while the presence or absence of colonies able to grow in the presence of Km and Cm are direct indications of the survivability of an exchange of the gene of interest for the Km^r selectable marker. Values are representative of three independent experiments.

sential functions of these two plasmids into new vectors with more acceptable selectable markers. The resulting insertion vector, pSABA-3 (Fig. 1), retains a partial pT181 rolling-circle origin of replication that is inert in gram-positive cells due to the lack of the corresponding *repC* gene. Also included was an Em^r gene to track the presence of the plasmid backbone, as well as a Km^r gene used as the core of the gene replacement constructions. Restriction sites on either side of the Km^r gene (Fig. 1) allowed insertion of 5' and 3' flanking sequences of a gene of interest to create a GRI plasmid. These flanking sequences directed homologous insertion of the GRI plasmid either immediately upstream or downstream of the gene of interest within the *B. anthracis* genome, while run-on expression from the Em^r and Km^r genes minimized any resulting polar effects on downstream genes. A second plasmid, pRX3-*repC*, was then introduced into confirmed GRI strains to deliver expression of the RepC protein, which inhibits DNA replication in any cells that retain genomic insertions of the partial pT181 origin (68). This provides a powerful and efficient counterselection for growth of only those cells that have managed to excise the pSABA-3 backbone by a second recombination event.

If the plasmid were excised using homology at the same side of the gene of interest into which it originally inserted, the gene would be restored to its original form and function and the resulting strain would essentially resemble the original UM23C1-1 background. An equally likely plasmid excision event can use homology on the side of the gene opposite the original site of insertion, which results in removal of the gene of interest in exchange for the pSABA-3 Km^r gene. Therefore, the ratio of the numbers of surviving Km^r and Km^s pRX3-*repC* transformants for each GRI strain is a direct indication of the essentiality of the particular gene of interest. The results for the *metS1*, *metS2*, *murB1*, and *murB2* genes are summarized in Table 4. For *murB1* and *metS2* GRI strains, roughly half of Cm^r pRX3-*repC* transformants were also found to be Km^r. PCR and sequencing analyses of the insertion loci confirmed that these Km^r colonies exchanged their *murB1* or *metS2* gene with the Km^r gene and that no other trace of the original allele or the pSABA-3 plasmid remained within the genome. Moreover, no significant difference in growth rate was observed for confirmed *murB1* and *metS2* gene replacement mutants in liquid growth medium (data not shown), which indicates that

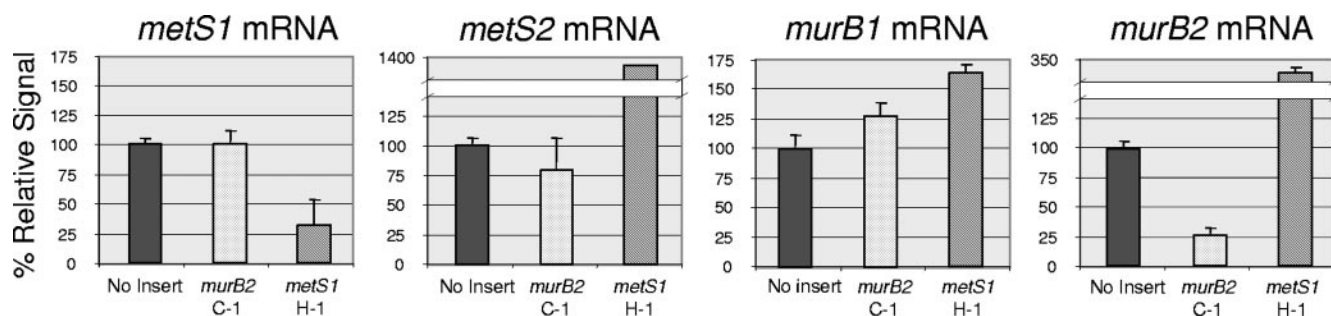


FIG. 2. Quantitation of mRNA by RT-PCR in response to expression of either *metS1* or *murB2* antisense RNA. *B. anthracis* cells transformed with vector pBAX-2 including the 96-bp *metS1* antisense fragment insert H-1 (5'-AATATAACTGGATCTACTACATTTCCTTTTGACTTAC TCATCTTTCCATCCTTCATTAATAAATCCAACCGTGAGCAAAGACTTTTTTCGGAAGAGGT-3'), the 114-bp *murB2* antisense fragment C-1 (5'-CCGCCAACTGAACCTGGAATACCACAAGCGAACTCAAGACCCGTTAAGTTATGGTCTAACGCAATACGTGATACGTC AAATTGCTGCACCGCACTGTGCTACAATGTGCTT-3'), or no fragment insert were grown cultured in MH Cm34 broth with 2% xylose. Total RNA was isolated from each strain after incubation at 37°C for 1 hour and then *metS1* and *murB2* transcripts were quantified by RT-PCR as described in Materials and Methods. Error bars represent standard deviations of data obtained from three replicate experiments.

these genes are dispensable for vegetative growth. In contrast, pRX3-*repC* transformations of *metS1* and *murB2* GRI strains resulted only in colony growth on solid medium containing Cm, while no growth was observed when Km was also present. This shows that replacement of either the *metS1* or *murB2* gene with the pSABA-3 Km^r gene is not a survivable event; thus, these genes are essential for *B. anthracis* growth.

Validation of antisense mechanism by RNA analysis. The gene replacement results described above provide independent and corroborative evidence that the highly skewed ratio of antisense/sense fragment inserts seen for *metS1* and *murB2* is predictive of their essentiality, while a lack of xylose-dependent growth-inhibitory fragments (*murB1*) or a lack of bias in the antisense/sense orientation ratio of such fragments (*metS2*) is indicative of genes that are dispensable for cellular growth. To determine if the growth defects caused by conditional expression of any particular antisense-oriented DNA fragment are gene specific, we used real-time RT-PCR to measure the fate of *metS1*, *metS2*, *murB1*, and *murB2* gene transcripts in response to expression of *metS1* or *murB2* antisense RNA (Fig. 2). When expression of *murB2* antisense RNA was induced, only the level of *murB2* mRNA was significantly reduced compared to levels in a similarly treated strain containing the pRX3-33 base vector (no insert). In the reciprocal experiment, induction of *metS1* antisense RNA expression caused specific reduction only in *metS1* mRNA levels. Unexpectedly, *metS1* antisense induction caused significant increases in expression of *murB1*, *murB2*, and *metS2* transcripts (1.5, 3.5, and 14 times control levels, respectively), although the mechanism for these changes in expression remains unclear. Specific degradation of *metS1* or *murB2* transcripts was also observed in Northern blot experiments (data not shown). However, transcript levels from *metS2* or *murB1* were undetectable using this technique.

Expression of antisense RNA corresponding to an essential gene can hypersensitize *B. anthracis* to target-specific antibiotics. Antisense strains that caused specific degradation of *metS1* or *murB2* message and exhibited strong growth inhibition at minimal xylose concentrations were further tested for hypersensitivity to target-specific antibiotic drugs. Hypersensitivity to an antibiotic was measured as a shift in the dose-response curve at the IC₅₀ value, in the presence or absence of

xylose at xyl₅₀ concentrations. Figure 3 illustrates the growth response of the *metS1* antisense strain to four different antibiotics: the protein translation inhibitor erythromycin, the fatty acid biosynthesis inhibitor cerulenin, the cell wall biosynthesis inhibitor cloxacillin, and Rx-000019, a compound with known specificity to the gram-positive MetS enzyme (11, 19). Dose-response curves with and without xylose induction of antisense expression did not differ significantly for erythromycin, cerulenin, and cloxacillin, yet the IC₅₀ value of the xylose-affected dose response to Rx-000019 was consistently shifted to an eightfold-lower value than that seen for cells not treated with xylose.

Changes in the IC₅₀ of the *metS1* antisense strain in response to an expanded panel of antibacterials with defined MOA were compared to similar analyses of the *murB2* antisense strain (Fig. 4). The antibacterial panel included those that affect cell wall biosynthesis (cloxacillin, oxacillin, dicloxacillin, vancomycin, and fosfomycin), ribosome function (clarithromycin, erythromycin, tetracycline, gentamicin, and kanamycin), fatty acid biosynthesis (irgasan and cerulenin), RNA polymerase (rifampin), DNA replication (ciprofloxacin and novobiocin), and methionyl tRNA synthetase (Rx-000019). Induction of *metS1* antisense expression again caused a significant shift in dose response to Rx-000019 but not to any of the other antibacterials in the panel. Conversely, induction of *murB2* antisense caused no significant shift in IC₅₀ for the Rx-000019 strain, nor for any of the other inhibitors with the exception of those that target cell wall biosynthesis. Strong xylose-dependent IC₅₀ shifts were observed for the beta-lactam drugs cloxacillin, oxacillin, and dicloxacillin. Although these compounds do not directly target the MurB enzyme, they do target enzymes involved in the later and final steps in cell wall biosynthesis. Only a modest twofold shift was observed for the MurA inhibitor fosfomycin, while no shift was seen for vancomycin.

DISCUSSION

Antisense RNA has proven to be a useful tool for experimental reduction of gene expression in *S. aureus* (20, 31–34, 37, 70), as well as in other bacteria (14, 48, 56, 61–65). This has

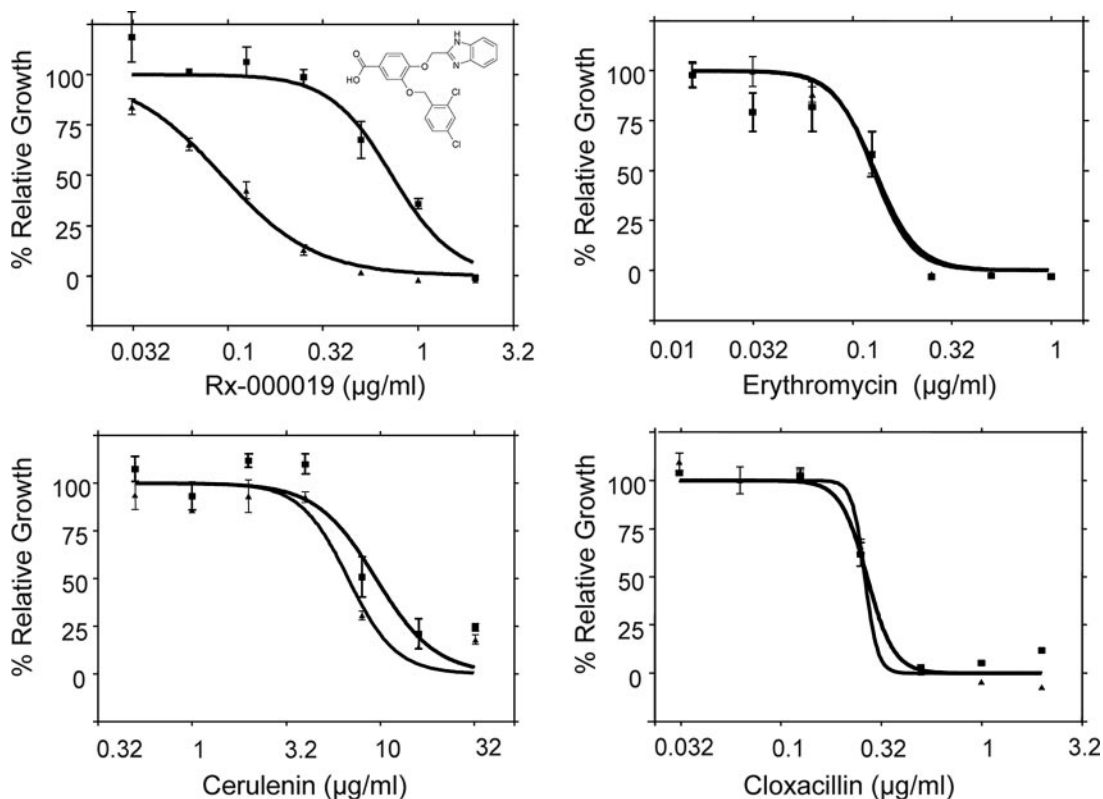


FIG. 3. Dose-response curves of various antibacterials on cellular growth of the *B. anthracis metS1* H-1 antisense strain, in response to the presence or absence of xylose. Concentrations of antibacterial compounds were included at seven twofold serial dilutions, starting at 2× the MIC measured for UM23C1-1 cells containing the pBAX-2 base vector. Growth was conducted in quadruplicate 50-µl volumes for each antibacterial concentration in 384-well plates, in both the presence and the absence of xylose at a 60 mM concentration. After 15 h of incubation at 37°C, the OD₆₀₀ was determined using a TECAN GENios plate reader. Quadruplicate values for each drug condition were averaged and then normalized to growth levels without added drug. Resulting normalized data were plotted to establish dose-response curves of growth in the presence (▲) or absence (■) of xylose. The structure of Rx-000019 (11, 19) is shown as an inset.

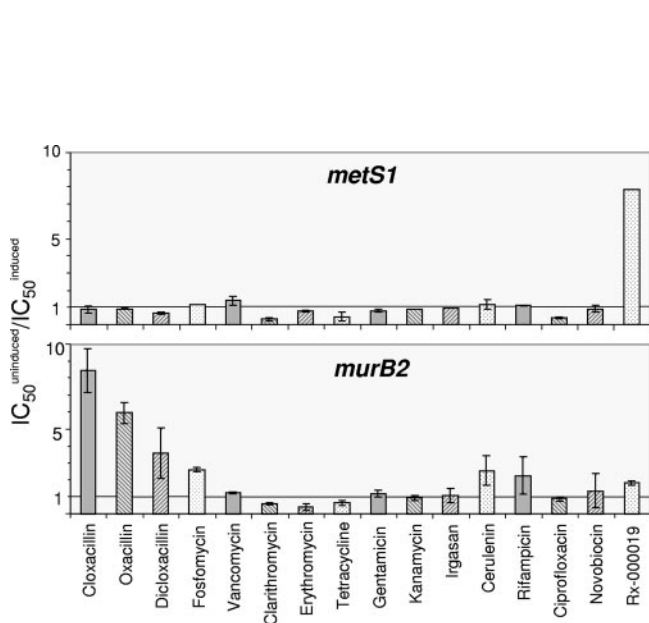


FIG. 4. Xylose-dependent shifts in dose responses to various antibacterials for the *metS1* H-1 and *murB2* C-1 antisense strains. Shifts for each antibacterial agent are presented as the ratio of IC₅₀ values for uninduced cells over those of 60 mM xylose-induced cells.

largely been accomplished by linking gene fragments in the antisense orientation to a plasmid-borne inducible promoter. Introducing these plasmid constructs into a bacterium and then growing them in the presence of an inducer compound can cause phenotypic changes characteristic of null mutations in a target gene by specifically blocking translation of the target gene mRNA. When a target gene is essential for viability, antisense RNA expression can result in attenuation of growth rate (20, 32, 34), a phenotype that is reminiscent of target-specific antibacterial drugs. Indeed, the growth of cells expressing antisense RNA to a particular essential gene of interest becomes hypersensitized to the further addition of antibacterials that act as inhibitors of the affected gene product (20, 33). Recently, antisense-induced growth hypersensitivity was used as a live cell MOA assay to detect novel antimicrobial compounds that specifically inhibit fatty acid biosynthesis (47, 66, 71).

Here we have presented evidence that conditionally expressed antisense RNA is efficacious for targeted reduction of gene expression in the biowarfare-relevant pathogen *B. anthracis*. We developed a library screening method that thoroughly analyzes fragments of a single gene of interest for those that inhibit growth when expressed from a plasmid-borne conditional promoter. For this purpose, we constructed a xylose-

responsive expression plasmid, pBAX-2, which includes the *xylR* gene and *xylA* promoter of *B. subtilis* (7, 23, 24) in pRX3-33, a *B. anthracis*/*E. coli* shuttle vector. We used the pBAX-2 vector as a plasmid background to construct DNA fragment libraries of individual genes of interest. These libraries were then introduced into *B. anthracis* to screen for individual clones that inhibited growth only in the presence of xylose. To evaluate this method, we chose to build pBAX-2-based fragment libraries of *B. anthracis* genes encoding two highly valued targets for novel antibacterial development: methionyl-tRNA synthetase (MetS; protein translation) and UDP-*N*-acetylenolpyruvoylglucosamine reductase (MurB; peptidoglycan biosynthesis). *B. anthracis* is unusual in that its genome contains two paralogs each of the *metS* and *murB* genes, which in most other bacteria occur as single essential loci.

Without prior knowledge of the relative essentiality of the *B. anthracis metS* and *murB* paralogs, we conducted empirical screens of each of the four genes for DNA fragments that cause target-specific growth inhibition. Screens of the *metS1* and *murB2* libraries yielded numerous growth-inhibitory gene fragments, nearly all of which were in the antisense orientation. The high antisense/sense insertion ratios observed for the *metS1* and *murB2* screens strongly correlated with the essentiality of these alleles, as demonstrated by gene replacement mutagenesis, and were an indication that the observed growth-inhibitory phenotypes were due to target-specific reduction in mRNA levels, as confirmed by real time RT-PCR measurements. Conversely, either the lack of insertion orientation bias among growth-inhibitory fragments or simply the lack of growth-inhibitory fragments in either orientation was observed for the demonstrably nonessential *metS2* and *murB1* alleles. The extreme antisense bias exhibited in the screens of the essential alleles was remarkable, especially with the expectation that this screen could also detect "sense" fragments that acted in a specific dominant-negative manner. This apparent "crowding out" of dominant-negative sense fragments by antisense fragments could signify that antisense expression is more generally able to result in target-specific functional attenuation under these conditions.

The essentiality of only one of each of the *metS* and *murB* paralog genes was unexpected based upon essentiality determinations for paralog sets in other bacteria. In about 40% of clinical isolate strains of *Streptococcus pneumoniae*, resistance to MetS-specific antibiotics was traced to the presence of a second functionally expressed *metS* allele (25). Interestingly, the essential *S. pneumoniae metS2* gene is more closely related to the nonessential *metS2* allele of *B. anthracis* (65% direct identity on the amino acid level), and it has been suggested that *B. anthracis* was the source of the *S. pneumoniae* allele by a horizontal transfer event (10, 25). If so, it is unclear why this essential function was observed in *S. pneumoniae* yet is silent in *B. anthracis*. It has been suggested that the presence of the MetS2 enzyme in some *S. pneumoniae* strains might be due to the influence of an as-yet-undiscovered MetS-specific antibiotic factor in the clinical environment (25). Conversely, lack of such an environmental stimulus may have led to the degeneration of the *B. anthracis metS2* allele to a pseudogene (44). Or, it may yet function in an alternative and undefined cellular role that is beyond detection by methods used in this study.

As with the *metS* gene paralogs, our results show that only one of the two *murB* alleles of *B. anthracis* has an essential role in cell viability. However, the choice of the *murB2* gene as the functional allele is not expected when homologies with other essential *murB* orthologs are taken into consideration. Of the two *B. anthracis murB* paralogs, the nonessential *murB1* gene is more closely related to the single essential *murB* gene of *B. subtilis* (66% direct identity on the amino acid level) (13, 55). The *B. subtilis* gene is situated within the division/cell wall (*dcw*) gene cluster that coordinates the expression of a number of murein biosynthesis, cell division, and sporulation functions (55, 57). The arrangement of the *murB1* operon is highly conserved in *B. anthracis* (54). In contrast, the essential *murB2* gene of *B. anthracis* is expressed as a single monocistronic unit that is physically remote from other genes involved in cell wall biosynthesis or related functions. The MurB2 amino acid sequence is more similar to the MurB enzyme of *S. aureus* (60% identity) than to either the *B. subtilis* MurB or *B. anthracis* MurB1 enzymes (both about 38% identity) (Fig. 3), suggesting that the *murB2* gene was itself acquired from an extra-genomic source. Whatever the reason for the *murB* gene duplication in *B. anthracis* and related bacteria in the *Bacillus cereus* sensu lato group (29, 53), it remains unclear why *B. anthracis* has delegated essential function for vegetative growth to a gene that is at least ostensibly outside of coordinated cell wall/cell division regulation. Since the *B. subtilis* MurB enzyme has been shown to be involved in sporulation (55), the *B. anthracis* MurB1 enzyme may still retain this function within the pathogen's life cycle.

B. anthracis strains carrying antisense fragments that were shown to cause target-specific reduction of *metS1* or *murB2* expression were particularly sensitive to the added presence of antibiotic compounds that inhibit corresponding cellular functions. Induced expression of *metS* antisense specifically hypersensitized *B. anthracis* growth to Rx-000019, a MetS-specific antibiotic compound (11, 19), but not to antibiotics acting through any other mechanism. Similarly, strains expressing *murB2* antisense were hypersensitized to antibiotics that target cell wall-related functions (i.e., beta-lactams), but not to antibiotics that target other metabolic functions. This hypersensitivity implies that a decrease in MurB activity somehow results in an increased effectiveness of beta-lactam antibiotics. Interestingly, this type of synergistic relationship has also been observed in *S. aureus* when beta-lactams are combined with the MurA-specific antibiotic fosfomycin (42), as well as in beta-lactam sensitivity experiments in *S. aureus* and *B. subtilis* involving genetic reduction in Mur enzyme expression (22, 55, 59).

The phenotypic behavior of the *metS1* and *murB2* antisense strains to different antibacterials provides proof of concept for use of these experiments as MOA detection assays. We envision a broad panel of individual assays based on *B. anthracis* antisense strains affecting expression of other essential genes, such as those involved in DNA replication, fatty acid biosynthesis, RNA replication, and protein translation, as well as those encoding other tRNA synthetases and peptidoglycan biosynthesis enzymes. This empirical antisense fragment discovery and validation process allows selection of the most specific and effective antisense fragments for incorporation into these assays. With such a panel of facile MOA detection

tools, antimicrobial hit compounds from synthetic or from natural product sources can be evaluated for specificity to cellular targets of interest. Incorporation of this facile MOA assessment with traditional structure-activity relationship experiments could expand the number of potential lead antibacterials series that eventually reach the clinic as effective treatments of anthrax or infectious diseases caused by other gram-positive pathogens.

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