

The Relative Contributions of the Two *murA* Paralogs to Essential Peptidoglycan Biosynthesis in *B. anthracis*

R. HASELBECK, K. GC, D. Reyes, V. BROWN-DRIVER, M. STIDHAM, M. HILGERS, J. FINN
Rx3 Pharmaceuticals, San Diego, CA

Robert J. Haselbeck Ph.D.
 6310 Nancy Ridge Dr. Ste. 105
 San Diego, CA 92121
 (858) 452-0370 ext. 107
 rhaselbeck@rx3pharma.com

Abstract

Background: Low GC-content Gram-positive bacteria generally contain two paralogous genes for UDP-N-acetylglucosamine enolpyruvyl transferase (MurA), the first enzyme in peptidoglycan biosynthesis. In *Streptococcus pneumoniae*, deletion of either gene, but not both has been shown to be survivable (1), indicating that both enzymes contribute significantly to this essential function in whole cells. In other Gram-positive bacteria, including the biowarfare-relevant human pathogen *Bacillus anthracis*, the relative contributions of the two MurA paralogs to cell-wall biosynthesis is not as clear. Here we provide genetic and functional evidence that only one of the two *murA* genes of *B. anthracis* is essential for viability.

Methods: A screen for gene fragments of *B. anthracis murA1* and *murA2* that could cause growth inhibition when inducibly expressed in the antisense direction was conducted. The ability to obtain significant numbers of antisense strains for a particular gene implied its essentiality, which was independently corroborated by a novel gene replacement mutagenesis technique. Hypersensitivity of antisense strains to fosfomycin, the MurA-specific antibiotic, as well as to other drugs was tested.

Results: Fifteen growth inhibitory gene fragments, all in the antisense orientation, were found to map to the *murA1* gene. No growth inhibitory gene fragments were found in a similar screen of *murA2* gene fragments. Replacement of the *murA2* gene with a kanamycin resistance gene yielded viable mutant *B. anthracis* strains, while similar analysis of the *murA1* gene determined that it was essential for viability. Moderate expression levels of *murA1* antisense RNA caused specific growth hypersensitivity to fosfomycin as well as to beta-lactam drugs.

Conclusions: Two independent analyses confirmed the essentiality of only one of the two *murA* paralogs of *B. anthracis*. Reduction in expression of the *murA1* gene causes hypersensitivity to fosfomycin, despite the high intrinsic resistance of *B. anthracis* to this drug. A single essential protein improves the ease of using structure-based drug design to develop novel anti-MurA drugs against this important pathogen.

Results and Methods

A: The two *murA* genes of *B. anthracis* are unlinked and are each likely to be transcribed as monocistronic messages.

B: We performed empirical screens for antisense RNA clones for both *murA1* and for *murA2*, in manners similar to our approach on the paralogous gene pairs *murB1/2* and *metS1/2* of *B. anthracis* (4). Genes of interest were amplified from *B. anthracis* UM23C1-1 genomic DNA using oligonucleotide primers that include ~200 bp of 5' and 3' flanking sequences.

C: The amplification products were subjected to sonication to generate small fragments ranging between 100 and 400 bp in length.

D: Gene fragments from both *murA-1* and *murA-2* were then mixed with fragments of other cell-wall related genes and ligated into the *SmaI* site of the xylose-responsive expression vector pBAX-2 (4), without bias for insert orientation.

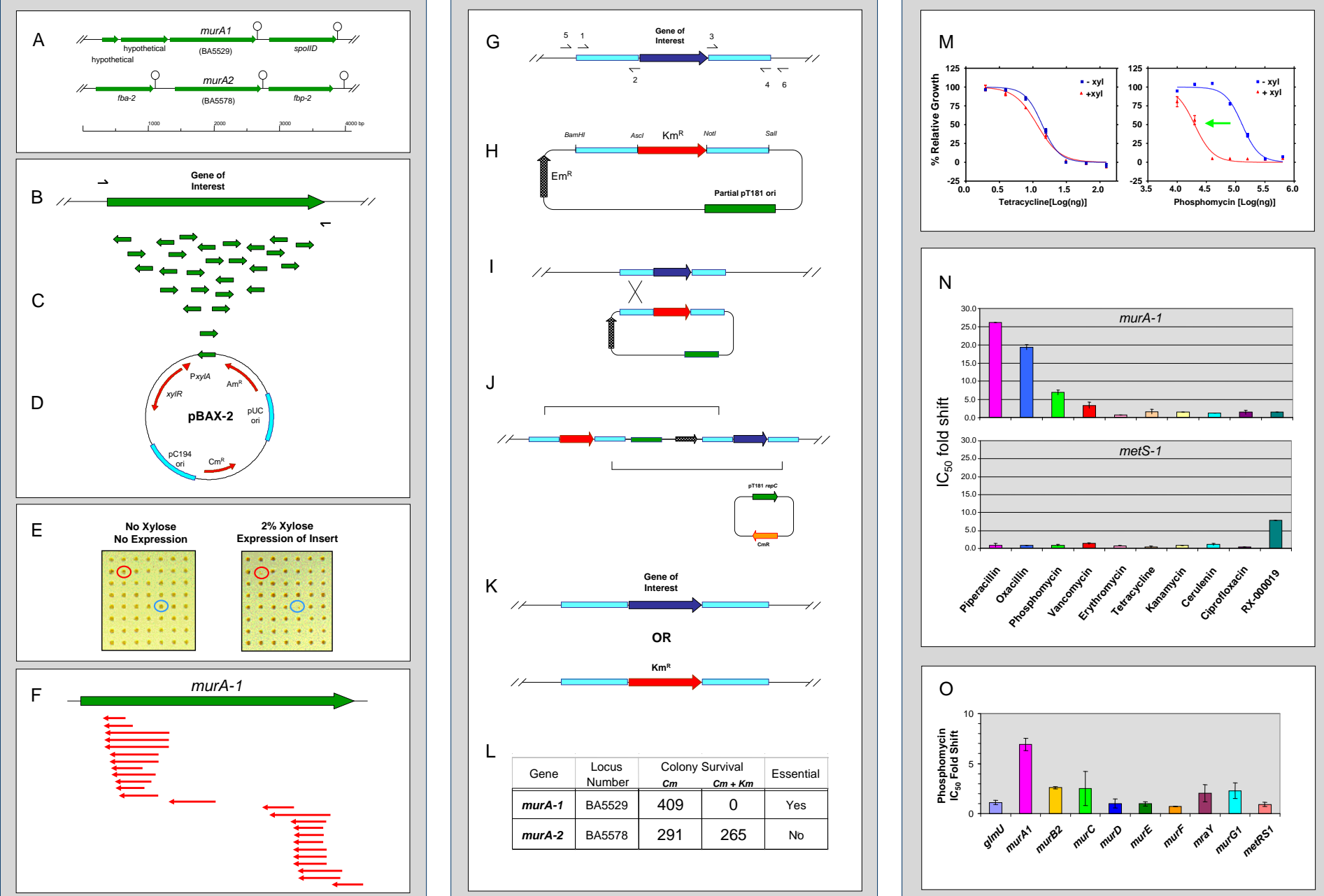
E: After introduction of the library into *B. anthracis* UM23C1-1, individual transformant colonies were screened for growth in the presence and in the absence of xylose at 2% final concentration. This growth sensitivity was reconfirmed in secondary tests.

F: Plasmids isolated from xylose-sensitive strains and the DNA sequence and orientation of the inserts was determined. All twenty-five growth sensitive isolates obtained for *murA-1* contained gene inserts in the antisense orientation, while no *murA-2* gene fragments were detected in this screen, suggesting that only *murA-1* is essential for viability.

G: A gene replacement strategy was developed for *B. anthracis* based on a technique originally described for use in *Staphylococcus aureus* (8). Oligonucleotide primers were designed for each gene to amplify the 5' and 3' flanking sequences (1-4), and for later use in genomic mapping (5,6).

H: The amplification products were inserted on either side of a kanamycin resistance (*Km^R*) gene within the insertion plasmid pSABA-3, which also includes an erythromycin resistance (*Em^R*) gene and an inert portion of the pT181 origin of replication.

Figures



Results and Methods (continued)

I: Completed KO plasmids were then transformed into *B. anthracis* UM23C1-1, selecting for colony growth on solid LB medium plus *Em* and *Km*. PCR was used to confirm single cross-over insertion of the KO plasmid either immediately upstream or downstream of the gene of interest.

J: Resulting insertion strains were then transformed by a second plasmid, pRX3-*repC*, which confers chloramphenicol resistance (*Cm^R*) and overexpresses pT181 *RepC*. Restoration of *RepC* expression in trans to the inserted pT181 origin is lethal, so only those strains that have excised the KO plasmid by a second recombination event (brackets) can survive this counterselection.

K: Excision of the pSABA-3 backbone is equally likely to restore the affected locus to its original state, or can result in a precise replacement of the targeted ORF with the *Km^R* gene.

L: Relative numbers of these two species were tested by plating pRX3-*repC* transformations on solid medium containing *Cm* as well as on medium containing both *Cm* and *Km*. Numerous robust *Cm^R + Km^R* resistant colonies were found for *murA-2* strains, indicating that exchange of the *murA-2* ORF with the *Km^R* gene is a survivable event. In contrast, no *murA-1* strains were able to survive on medium containing *Km*, indicating that this gene is indispensable for growth.

M: We chose a highly responsive *murA-1* antisense strain to develop a mode-of-action (MOA) assay for antibiotic compounds that are specific to *B. anthracis* MurA. The strain was subjected to growth in the presence of various antibiotics at a range of concentrations to generate dose response curves, either in the presence or absence of xylose at an apparent *IC₅₀* concentration. The dose response curve for the MurA-specific antibiotic fosfomycin dramatically shifts to lower effective concentrations in the presence of xylose and antisense RNA expression, while no such effect is seen for an unrelated antibiotic (e.g. tetracycline).

N: Dose response curve shifts were measured for a panel of different antibiotics in the presence and absence of antisense induction. The *murA-1* strain was compared with a *metS* antisense strain that has been shown to be specifically hypersensitized to antibiotics specific to methionyl-tRNA synthetase (2, 4). In addition to fosfomycin, the *murA-1* antisense strain was hypersensitized to beta-lactam drugs, synergy that has also been observed with fosfomycin (5) as well as with dial-down expression of other Mur enzymes (3, 4, 6, 7).

O: As expected, hypersensitivity to fosfomycin was primarily observed in the *murA-1* antisense clones, and less so in antisense strains representing other genes involved in peptidoglycan biosynthesis.

Conclusions

- Only the *murA-1* gene, and not the *murA-2* gene, is essential for viability of *B. anthracis*.
- Expressing *murA-1* antisense RNA specifically hypersensitizes *B. anthracis* cells to the MurA-specific antibiotic fosfomycin, and can synergize the effects of beta-lactam drugs.
- The *murA-1* antisense strain is a valuable MOA detection tool for novel MurA-specific antibiotics effective against *B. anthracis*.
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References

- Du et al. (2000). J Bacteriol 182:4146-52.
- Finn et al. (2001) 41st ICAAC-Chicago F-2140.
- Gardete et al. (2004). J Bacteriol 186:1705-13.
- Kedar G.C et al. (2005). 45th ICAAC-Washington, D.C. F-2075.
- Nakazawa et al. (2003) J Infect Chemother 9:304-9.
- Real et al. (2006) J Bacteriol 188:1721-32.
- Sobral et al. (2003). Microb Drug Resist 9:231-41.
- Xia et al. (1999) Plasmid 42:144-9.