077 (D)

Targeted Antisense for Essentiality Determination of Non-Mevalonate Isoprenoid Biosynthesis Genes in *Bacillus anthracis*

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Abstract

Introduction: Although the non-mevalonate isoprenoid biosynthesis pathway is conserved across many genera of bacteria including *B. anthracis*, *Y. pestis*, and *F. tularensis*, it is absent in humans. The six steps in the pathway are thus selective targets for the development of novel biodefense antibiotics. Previously, we have demonstrated the effectiveness of targeted inducible antisene expression in *B. anthracis* for essentiality determination and for drug discovery on genes involved in cell wall biosynthesis and protein synthesis (5). Here we show results of empirical screens for fragments of non-mevalonate pathway genes that cause growth inhibition due to post-transcriptional inhibition when inducibly expressed in the antisense orientation.

Methods: Random fragment libraries of eight genes involved in the non-mevalonate pathway were constructed in a xylose-controlled expression vector. Transformant strains that grew in the absence of xylose but failed to grow in the presence of xylose were identified for further characterization. This included DNA sequence analysis of the plasmid inserts as well as sensitization of the transformants to various antibiotics.

Results: Numerous growth-inhibitory gene fragments, all in the antisense orientation, were found to map within the *B. anthracis* genes for *dxs, dxr-2, ispD (ygbB), ispE (ychB), gcpE (ccpE)* and *lytB*. Growth-inhibitory fragments mapping to the *ispF (ygbP)* gene were equally likely to be in the sense or antisense orientation. No –growth-inhibitory fragments corresponding to the *dxr-1* paralog were identified.

Conclusions: The frequency and antisense orientation bias of growth inhibitory fragments isolated for the *dxs, dxr-2, ispD, ispE, gdpE*, and *lytB* genes are strong indications of the essentiality of these genes during normal growth of *B. anthracis*, as well as their suitability as novel antibiotic drug targets.

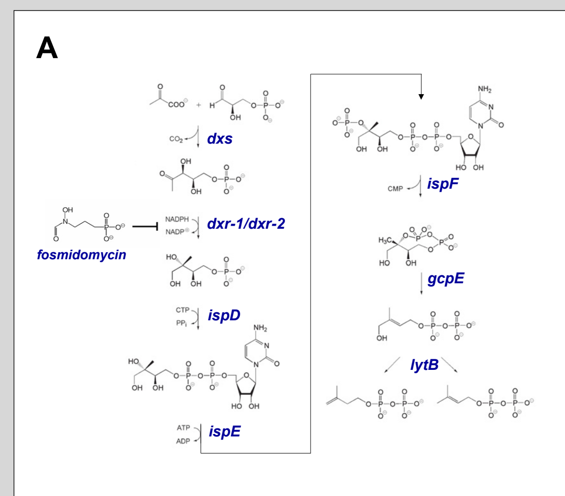
Results and Methods

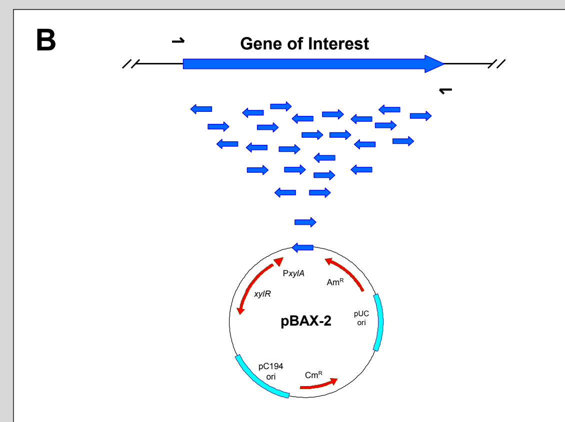
A: The non-mevalonate isoprenoid (NMI) biosynthesis pathway provides essential building blocks for fatty acid and terpenoid biosyntheses in many bacteria (2, 11), including *B. anthracis.* The first concerted step in the pathway is catalyzed by 1-deoxy-d-xylulose 5-phosphate reductoisomerase (Dxr), which is specifically inhibited by the antibiotic fosmidomycin (7). In the genome of *B. anthracis,* Dxr is apparently represented by two distinct loci (9); the relative contribution of these gene paralogs to overall Dxr expression is unknown.

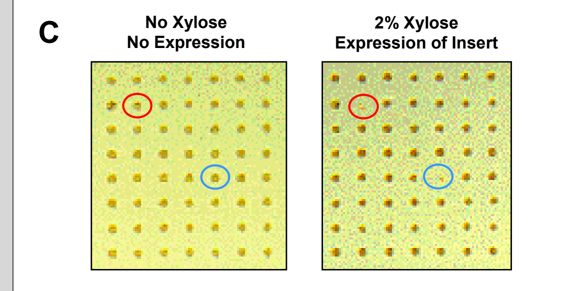
B: We conducted an empirical screen for NMI gene fragments that can block growth when conditionally expressed. Each gene was PCR amplified from genomic DNA prepared from *B. anthracis* strain UM23C1-1 (8). The PCR products were then subjected to sonication to generate fragments in the range of 200-600 bp in length. These fragments were then inserted immediately downstream of the xylose-inducible promoter of vector pBAX-2 (5) to create random-fragment gene libraries.

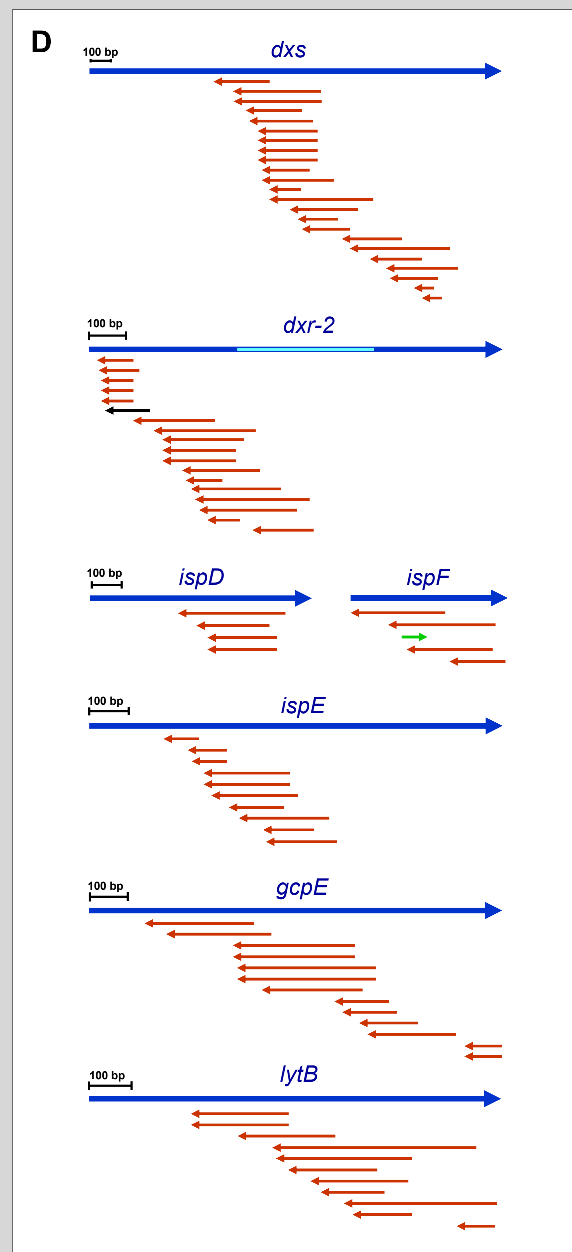
C. Library DNA was transformed into *B. anthracis* UM23C1-1 cells and resulting colonies were screened for the ability to grow on solid medium in the presence and absence of 2% xylose. The xylose-dependent growth sensitivity of colonies was confirmed in secondary repeat experiments. Gene fragment inserts were PCR amplified from xylose-sensitive colonies, subjected to DNA sequence analysis, and mapped to their respective NMI gene.

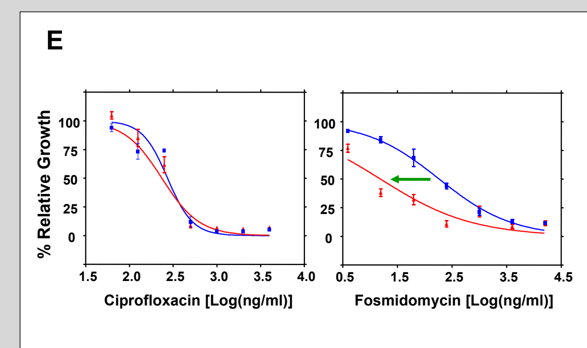
Figures

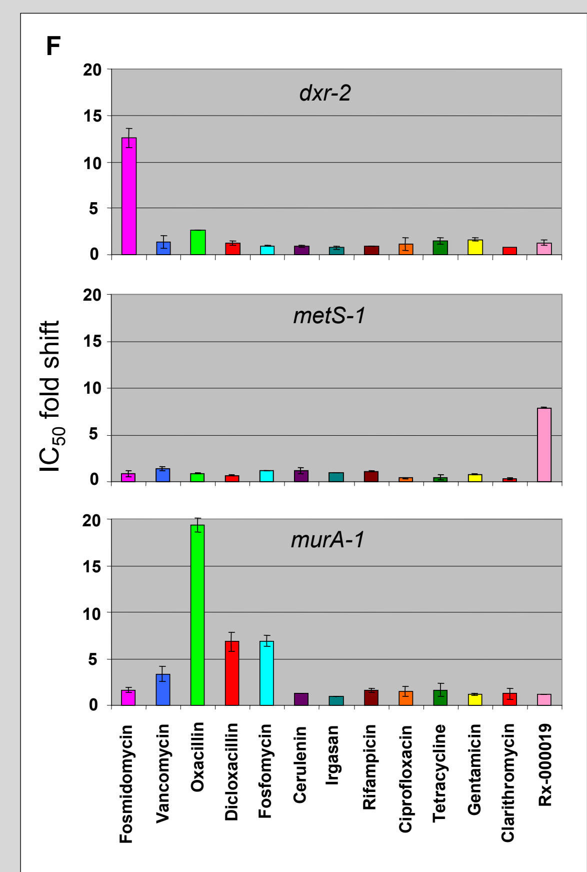












Results and Methods (continued)

D: The *dxs, dxr-2, ispD, ispE, ispF, gcpE,* and *lytB* genes were all represented by numerous growth-inhibitory fragments, all but one in the antisense orientation. In contrast, fragment inserts in randomly sampled library recombinants were equally likely to be in the sense or antisense orientation (data not shown). No growth-inhibitory fragments in either orientation were found to correlate with the *dxr-1* paralog. In previous work we found that large antisense representation in these screens correlated with gene essentiality (5), suggesting that the *dxr-1* gene is dispensable for growth. A region of high DNA sequence identity (71%) between the *two paralogs* is marked in light blue.

E: We chose a highly responsive *dxr-2* antisense strain (123 bp gene insert, black arrow in Fig. D) to develop a mode-of-action (MOA) assay for antibiotic compounds that are specific against *B. anthracis* Dxr function. The strain was cultured with various antibiotics at a range of concentrations to generate dose response curves, either in the presence or absence of IC50 xylose. Antisense expression shifted the dose response of the Dxr-specific antibiotic fosmidomycin to lower effective concentrations, while no such effect was seen for an unrelated antibiotic (e.g. ciprofloxacin). Fosmidomycin MIC against *B. anthracis* UM23C1-1 was 8 μg/ml.

F: Xylose-dependent shifts in IC50 values (green arrow, Fig. E) were determined for the *dxr-2* antisense strain against a panel of different antibiotics. These values were compared to those obtained for antisense strains previously developed for *B. anthracis metS-1* (5) and *murA-1* (10) . The *dxr-2* strain was hypersensitized to fosmidomycin, but not to antibiotics that affect cell-wall biosynthesis, fatty acid biosynthesis, DNA or RNA replication, or protein translation. In contrast, the *metS-1* antisense strain responded only to the MetS-specific antibiotic compound Rx-000019 (1, 3). The *murA-1* antisense strain was hypersensitized to the MurA-specific antibiotic fosfomycin, and also displayed characteristic synergy with beta-lactam drugs (4-6, 12).

Conclusions

- Antisense response and fosmidomycin sensitivity demonstrate the validity of NMI biosynthesis as a target for development of antibiotics effective against *B. anthracis*.
- Of the two *B. anthracis dxr* loci, only one appears to be essential. We have previously demonstrated similar functional relationships between paralogous *metS, murA*, and *murB* gene pairs in this organism (5, 10) .
- NMI-specific antisense strains can be used to screen for novel antibiotics that specifically target this important biosynthetic pathway.
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