

Revised Abstract

Background: Inducible expression of gene fragments in antisense orientation has been shown to specifically inhibit expression of essential genes in *Staphylococcus aureus* [1, 2]. Here we demonstrate that this powerful expression modulation tool can also be applied to the discovery of small molecules active against the biowarfare pathogen *Bacillus anthracis*. Lower expression levels of essential genes hypersensitize bacteria to the effects of target-specific inhibitors, thereby enabling target validation, screening and mode of action (MOA) determination.

Methods: An *E. coli*-*B. anthracis* shuttle vector was constructed with a xylose inducible promoter system. Essential genes of interest were PCR amplified, fragmented by sonication, and subcloned under the control of the inducible promoter to create random fragment libraries. Gene fragments that confer *B. anthracis* growth sensitivity only under inducing conditions were empirically determined; >90% of these were found to be in the antisense orientation to the promoter.

Results: Target specificity of antisense-based growth inhibition was demonstrated. A *B. anthracis* antisense strain targeting methionyl tRNA Synthetase (*metRS-1*) was shown to be specifically sensitized to a *MetRS*-specific compound Rx-000019 [10] and not to antibiotics that affect other functions. Likewise, we observed synergistic effects from cell wall synthesis inhibitors on the strain containing a *murB-2* antisense. As another example, a *B. anthracis* strain containing a *dfrA* antisense fragment was shown to confer growth sensitivity only in thymidine-depleted growth medium, a phenotype that is characteristic of *dfrA* null mutations [3]. Antisense specificity was confirmed by experiments designed to detect the amount of mRNA degradation due to antisense induction of a specific gene fragment.

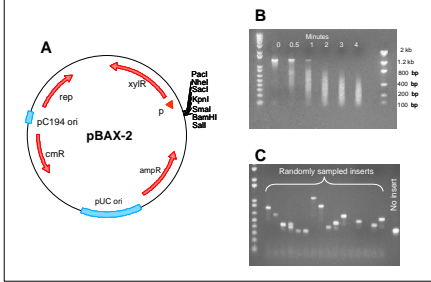
Conclusions: Inducible antisense can be used to specifically attenuate and titrate expression of key essential gene targets in *B. anthracis*. Lowered target gene expression can hypersensitize *B. anthracis* to the effects of small molecules that are specific inhibitors of the target. The application of this technique to *B. anthracis* enables target validation, MOA determination and target-specific drug screens which can accelerate the discovery of antimicrobials against this important pathogen. This work was supported by NIH SBIR grant R44 AI530009-02.

Results

- In transformed *B. anthracis* cells, incidence of sense and antisense insert orientations relative to promoter were found to be roughly equal prior to xylose induction.
- Upon induction with xylose, growth sensitive *B. anthracis* strains predominantly contained clones in the antisense orientation (Figure 2).
- Antisense rate for *metRS-1* and *murB-2* approached 100%; these genes were proven to be essential by standard gene-replacement mutagenesis (Figure 2).
- Antisense rate for *metRS-2* was 47%, while *murB-1* screen failed to produce any growth sensitive clones; these genes were shown to be non-essential by gene-replacement mutagenesis (Figure 2).
- Antisense against *metRS-1* and *murB-2* reduced measurable levels of their respective transcripts, but not that of their paralogs (Figure 3). Rather than being reduced, *metRS-2* mRNA increased 10 fold in the *metRS-1* AS strain relative to vector only.
- Moderately induced cells of antisense strains in *metRS-1* and *murB-2* were specifically hypersensitized only in the presence of specific antimicrobial compounds (Figure 4, 5).
- Antisense to *murB-2* hypersensitizes *B. anthracis* to beta-lactam antibiotics (Figure 5); this has also been observed for strains attenuated for *murE* and *murF* expression [4, 5].
- Moderate induction of *dfrA* but not *uppS* antisense strains caused thymidine auxotrophy (Figure 6), a characteristic of *dfrA* mutant strains [3].

Results

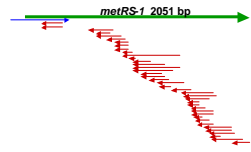
Figure 1: Library Construction



- pBAX-2 contains *xyIR*/AB expression system from *B. subtilis* (A).
- Sonicated gene fragments (B) were cloned into the *SmaI* site of pBAX-2.
- PCR amplification of inserts gauged relative insert size (C) and facilitated identification by DNA sequencing.

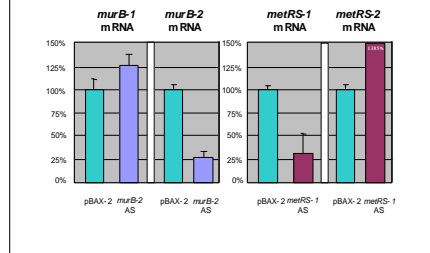
Figure 2: Characterizing Growth-Inhibitory Gene Fragments

Antisense Strain	Total CFU	Growth Sensitive	Antisense	Sense	Surviving KO CFU
<i>metRS-1</i>	2208	40	39	1	no
<i>metRS-2</i>	2400	19	9	10	yes
<i>murB-1</i>	2118	0	0	0	yes
<i>murB-2</i>	2024	26	26	0	no
<i>uppS</i>	2150	26	23	3	nd
<i>dfrA</i>	2304	21	15	6	nd



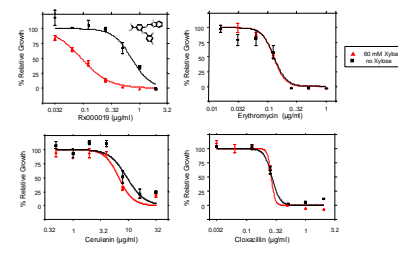
- Growth inhibitory inserts were predominantly in the antisense orientation for essential *metRS* and *murB* orthologs, as well as for *dfrA* and *uppS*.
- Non-essential *metRS* and *murB* orthologs yielded either equal numbers of sense and antisense inserts among growth sensitive clones or no growth sensitive clones at all.
- Gene replacement mutagenesis (KO) confirmed essentiality of *metRS* and *murB* orthologs as measured by KO survivability.

Figure 3: Fate of *murB* and *metRS* mRNAs upon expression of *murB-2* and *metRS-1* antisense



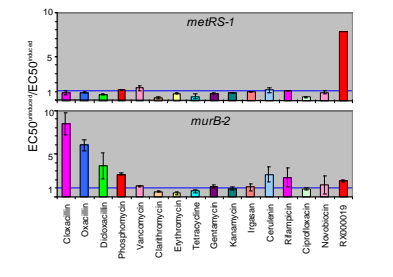
- Total RNA was purified from cultures taken 1 hour after xylose induction at time point zero (0.1 OD625).
- 3.75ng of total RNA was used per reaction for RT-PCR with *metRS-1*, *metRS-2*, *murB-1*, or *murB-2* specific primer sets.
- Rather than decreasing, levels of *metRS-2* message increased over 10-fold (as indicated in white) relative to the pBAX-2 containing control strain.

Figure 4: *Bacillus anthracis* growth response to four antibiotic compounds, as affected by induction of *metRS-1* antisense expression.



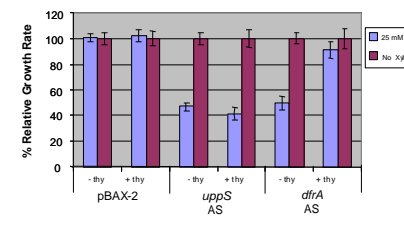
- B. anthracis* *metRS1* antisense strain was cultured in the presence (▲) or absence (■) of 60 mM xylose, and increasing concentrations of antibiotic.
- An antisense-dependent 8-fold downward shift in EC50 value was observed for the *metRS* specific compound Rx000019 [10].
- No significant shifts were observed for the translation inhibitor erythromycin, the fatty acid synthesis inhibitor cerulenin, or the PBP inhibitor cloxacillin

Figure 5: Antibiotic Sensitivity of *metRS-1* and *murB-2* Antisense Strains



- An antibiotic panel containing 16 drugs was screened against two antisense strains, *murB-2* and *metRS-1*.
- murB-2* antisense caused a synergistic shift in EC50 values for beta-lactam antibiotics.
- Compound Rx-000019 [10] caused a specific hypersensitivity effect only on the *metRS-1* antisense strain.

Figure 6: Growth deficiency of *B. anthracis* *dfrA* (DHFR) antisense strain is rescued by added thymidine



- B. anthracis* antisense strains of *dfrA*, and *uppS* were compared to base vector pBAX-2.
- Strains were cultured in thymidine-poor Mueller-Hinton II cation adjusted media. Antisense was either uninduced (■) or induced with 25 mM xylose (●).
- Thymidine added to growth media was sufficient to support auxotrophy of *dfrA* but not *uppS*. Added thymidine did not affect growth deficiency of a *uppS* antisense strain, but rescued the *dfrA* from antisense down regulation.

Materials and Methods

Vector Development

- An expression shuttle vector, denoted pBAX-2 (Figure 1A) was constructed.
- Includes replicative functions and chloramphenicol resistance gene of pC194 [6].
- Includes *E. coli* replicative functions and ampicillin resistance gene of pLEX5B [7].
- Includes *Bacillus subtilis* *xyIR* gene and *XylA* promoter derived from vector pSWEET-bgaB [8].

Library Construction and Screening

- Target genes were PCR amplified, fractionated by sonication (Figure 1B), end-polished, ligated into the *SmaI* site of pBAX-2, and rescued by transformation in *E. coli* DH5 α .
- Resulting libraries were amplified in *dam-/dcm-* *E. coli* INV110 (Invitrogen).
- Amplified library DNA was electroporated into *B. anthracis* plasmid-less strain UM23C1-1 [9].
- Randomly selected transformants were tested for insert size by PCR (Figure 1C). Range of insert sizes was between 100-400 bp.
- About 2000 resulting colonies per library were screened for growth sensitivity in BHI medium with or without added xylose inducer at 2% final concentration.
- Plasmid DNA from these colonies was analyzed by DNA sequencing to determine the sequence and orientation relative to the xylose inducible promoter and to the gene of interest (Figure 2).

Measurement of mRNA levels by RT-PCR

- The fates of particular gene transcripts in response to antisense induction were measured by Taqman Real Time RT-PCR (ABI) (Figure 3).
- Total RNA was purified from UM23C1-1 cells transformed with pBAX-2 base vector and with *metRS-1* and *murB-2* antisense clones after one hour of xylose induction.
- Data was normalized to 16S rRNA RT-PCR values for each sample.
- Determination of Antisense-based drug hypersensitivity**
- The antisense strains were grown under various doses of test compounds in the presence or absence of intermediate levels of xylose inducer.
- Drug dose responses were compared in +/- xylose conditions to determine the shift in EC50, an indication of test-compound specificity (Figure 4).
- EC50 fold shifts for each test compound was determined by dividing uninduced EC50 values by induced EC50 values (Figure 5).

Effect of *dfrA* antisense on thymidine auxotrophy

- Antisense strains representing *dfrA* and *uppS* were compared for growth in the presence and absence of thymidine (Figure 6).
- Cells were cultured in thymidine-poor Mueller-Hinton II cation adjusted medium.
- Xylose was either withheld or provided at 25 mM.
- Thymidine was either withheld or provided at 50 μ g/ml.

Conclusions

- Target-directed antisense specifically reduces gene expression in *Bacillus anthracis*.
- Resulting growth sensitivity phenotypes and specific effects on expression serve to validate essentiality of antibiotic target genes.
- metRS-1* and *murB-2*, but not paralogs *metRS-2* and *murB-1*, are essential for *B. anthracis* growth as determined by antisense and gene replacement mutagenesis.
- Antisense expression specifically hypersensitizes *B. anthracis* strains to antimicrobials that are on-target.
- Antisense-based hypersensitivity assays show great promise as facile MOA and screening tools.

References

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