

Specific Synergistic Effect of Mur Pathway Antisense Clones of *Bacillus anthracis* Against β -Lactams

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Abstract

Background: We have previously demonstrated the utility of antisense as a mode of action tool (ICAC 2005-F2075). We have also reported that *B. anthracis* transformed with inducible *murB* antisense is selectively sensitized to β -lactams even though β -lactams do not target *MurB*. Here we report that the sensitization to β -lactams extends to antisense clones for other genes in the Mur biosynthetic pathway.

Methods: *B. anthracis* Mur pathway antisense clones for *glmU*, *murA1*, *murB2*, *murC*, *murD*, *murE*, *murF*, *murY*, and *murG1* were identified after cloning random fragments of these genes into a xylose-inducible vector and transforming the library into an avirulent *B. anthracis* strain. Xylose sensitive clones were selected and sequenced. Antisense clones were tested for hypersensitization to a panel of inhibitors with different mechanisms of action.

Results: Antisense clones of each of the 9 genes in the Mur biosynthetic pathway were differentially inhibited by β -lactams in the presence of the antisense-inducer. In general, the antisense clones showed significantly greater inhibition by cephalosporins than penicillins. This predicts a high level of synergy between cephalosporins and Mur pathway antibacterials genes. There are interesting patterns between different β -lactams and different targets. None of these antisense clones showed any hypersensitivity to compounds unrelated to cell wall biosynthesis.

Conclusions: β -lactams selectively synergize Mur pathway antisense clones. In principle, this data will allow for the selection of optimized combinations of β -lactams and Mur pathway inhibitors.

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Materials and Methods

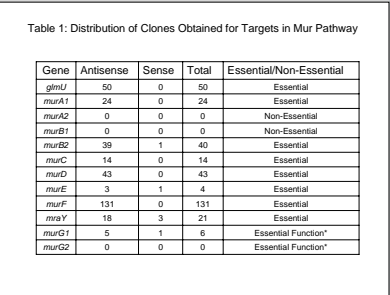
Plasmids and Strains: *Bacillus anthracis* strain, UM23C1-1, was used as the screening strain (11) pBAX2 (1, 2, 3) is an antisense expression vector, which has xylose inducible promoter derived from *Bacillus subtilis*. *E. coli* strains: *DH5a* and *INV110 (dam1 dcm)* (Invitrogen) were used as intermediate strains for library manipulation.

Media and Growth Conditions: *E. coli* strains were grown in LB broth and agar plates and transformants were selected in the presence of carbenicillin at 100 ug/ml. *B. anthracis* strain UM23C1-1 was grown in Brain Heart Infusion (BHI) broth or agar plates with or without 10 μ g/ml chloramphenicol.

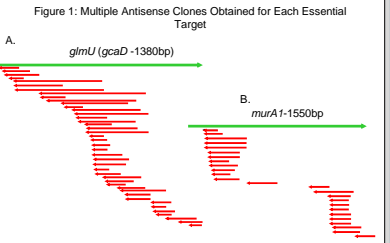
Antisense Library Construction and Screening: *B. anthracis* genes: *murA1*, *murA2*, *murB1*, *murB2*, *murC*, *murD*, *murE*, *murF*, *murY* and *murG1*, *murG2* were PCR amplified. The PCR products were combined in equal proportion, random fragments were generated by sonication, and the DNA ends were polished with T4 polymerase and Klenow fragment. These random library of genes were cloned into small site of pBAX2 cloning vector and transformed into DH5a cells. The library was passed through INV110 strain and subsequently transformed into *B. anthracis* by electroporation. Clones sensitive to 2% xylose were selected and plasmid inserts were sequenced for validation of antisense.

Specific Hypersensitivity Testing: Serial dilutions of known drugs were tested against antisense strains in the presence of slightly growth inhibitory concentrations of xylose (*glmU*=50mM, *murA1*=40mM, *murB2*=40mM, *murC*=60mM, *murD*=40mM, *murE*=20mM, *murF*=20mM, *murY*=20mM, *murG1*=40mM) as well as without xylose in BHI broth with chloramphenicol at 34 μ g/ml. One representative antisense clone for each gene was used to determine growth inhibition dose response curves for a panel of antibacterial drugs. The IC_{50} shift is defined as the ratio of the 50% growth inhibition concentration for the drug in the absence and presence of xylose.

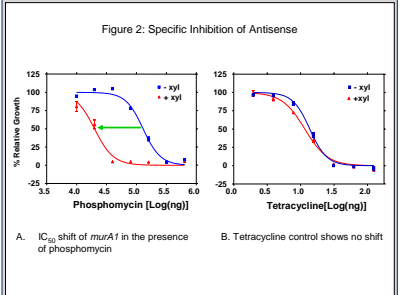
Results



- Only essential genes yielded antisense clones
- Both *murA2* and *murB1* were found to be non-essential and did not yield any antisense clones
- *murG1** and *murG2** can substitute for each other (unpublished knock-out data)
- Only *murG1* yielded xylose sensitive clones in our screening



- Multiple antisense clones were obtained for *murA1*, *murB2*, *murC*, *murD*, *murE*, *murF*, *murY* and *murG1*
- Some genes, such as *glmU* and *murF*, yielded antisense clones that spanned the ORF
- Other genes yielded antisense hot spots on the ORF

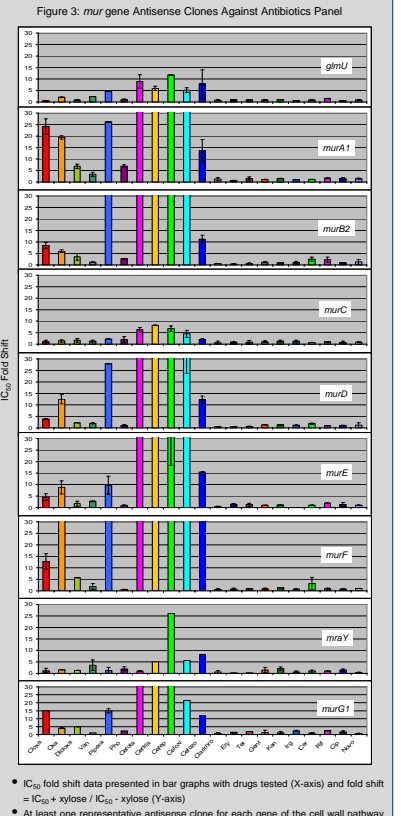


- Drug dose response curves were plotted in the presence (▲) or absence (■) of 40 mM xylose, and increasing concentrations of antibiotic
- No significant shift was observed for tetracycline. An antisense-dependent 6.5-fold shift in IC_{50} was observed for phosphomycin

Table 2: Antibiotics Panel Against *mur* gene Antisense Clones

Antibiotic	Target	<i>glmU</i>	<i>murA1</i>	<i>murB2</i>	<i>murC</i>	<i>murD</i>	<i>murE</i>	<i>murF</i>	<i>murY</i>	<i>murG1</i>
Cloxacillin	PBP	0.3	24.2	8.3	1.1	3.8	4.5	12.1	1.2	10.8
Oxacillin	PBP	2.1	19.4	6.0	1.4	12.5	8.3	37.2	1.6	4.0
Dicloxacillin	PBP	0.9	6.8	3.6	1.7	2.2	1.7	5.6	1.3	4.9
Vancomycin	PBP	2.4	3.4	1.2	1.3	2.0	2.7	1.9	3.6	1.1
Rifampicin	RpoB	4.8	26.2	>30	>20	27.8	9.7	37.5	1.3	14.8
Phenoxymethylpenicillin	MurA	1.1	6.8	2.8	2.0	1.0	1.0	0.7	2.0	2.3
ColistinA	PBP	9.0	>30	>30	6.3	>30	>30	>30	1.0	>30
ColistinB	PBP	6.0	>30	>30	8.2	>30	>30	>30	5.0	>30
ColistinC	PBP	11.8	>30	>30	6.7	>30	>30	>30	26	>30
ColistinD	PBP	5.2	>30	>30	4.5	>30	>30	>30	5.6	21.4
ColistinE	PBP	7.8	13.6	NA	21	12.2	15.5	>30	8.2	11.9
Gletothromycin	Ribosome	0.8	1.3	0.6	0.7	0.5	0.6	0.7	0.2	0.7
Erythromycin	Ribosome	0.9	0.7	0.4	0.6	0.6	0.5	0.6	0.2	1.6
Tetracycline	Ribosome	0.5	1.3	0.7	0.9	0.7	1.3	0.5	0.3	1.9
Gentamicin	Ribosome	0.9	1.2	1.2	1.1	1.4	1.0	1.0	1.4	1.4
Kanamycin	Ribosome	1.1	1.0	0.9	1.3	1.2	1.3	2.1	1.2	1.8
Nalidixic acid	FDX1	0.8	1.0	1.1	1.3	1.2	1.0	0.8	0.6	2.3
Clavulanic acid	FDX2	0.9	1.2	2.5	0.8	1.8	1.1	3.2	1.0	1.0
Rifampicin	rRNA	1.5	1.7	2.3	1.0	1.0	2.0	1.0	1.0	2.4
Ciprofloxacin	DNA gyrase	0.6	1.5	0.9	0.8	1.0	1.4	0.8	1.5	1.8
Neobornon	DNA gyrase	0.9	1.5	1.3	0.9	1.1	1.0	1.0	0.4	0.7

- IC_{50} fold shift values of known drugs against antisense clones are presented in the table
- Values more than 30 fold are given as >30



Results

Antisense clones for the following *B. anthracis* Mur pathway genes were screened: *glmU* (*gcaD*), *murA1*, *murA2*, *murB1*, *murB2*, *murC*, *murD*, *murE*, *murF*, *murY*, *murG1* and *murG2*. Even though the amount of DNA from each gene in the library was roughly equal, different numbers of antisense clones were identified for the various genes (Table 1). The different numbers of clones may indicate that each gene has intrinsically different susceptibilities to antisense inactivation. The location of antisense fragments occurred across the entire ORF for two of the genes *glmU* and *murF*. However, *murA1*, *murB2*, *murC*, *murD*, *murE*, *murY* and *murG1* genes yielded antisense fragments acting only at certain hot spots (Figure 1). The screening for clones with growth-inhibition in the presence of xylose indicated the essentiality of particular genes in *Bacillus anthracis* where only the essential gene would yield growth sensitive phenotypes. *murA2*, *murB1* and *murG2* did not yield any significant antisense clones indicating the non-essentiality of these genes in *B. anthracis* (Table 1). Our unpublished knock-out data suggested that *murG1* and *murG2* function can substitute each other. This finding is similar to the literature results from *murA1* and *murA2* in *Streptococcus pneumoniae* (4).

An antibiotic panel study against selected antisense clones demonstrated significant hypersensitivity to a number to PBP inhibitors, especially cephalosporins and to a lesser extent penicillins (Table 2 & Figure 3). None of these clones were hypersensitive to antibiotics that target other cellular pathways such as protein synthesis, DNA synthesis, RNA synthesis, or fatty acid synthesis. These results indicate that inhibition of any step in the Mur pathway leads to increased susceptibility to PBP inhibitors, especially cephalosporins. This type of hypersensitivity to PBP inhibitors was also observed in attenuated *murE* and *murF* strains (7, 8). In the present case, antisense induction was used to partially inhibit steps in the pathway. We anticipate that chemical inhibitors of the individual Mur steps in the pathway will also result in significant synergy with PBP inhibitors.

Conclusions

- Antisense clones for each step in the Mur pathway were obtained, confirming that each step represents an essential function in *Bacillus anthracis*
- Under antisense-induced conditions, clones for each gene in the pathway were hypersensitive to PBP inhibitors, especially cephalosporins
- The data suggest that inhibitors of Mur pathway enzymes will be highly synergistic with PBP inhibitors

References

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