Minimum Inhibitory Concentration Assays for Multidrug Resistant Gram-Negative Bacterial Pathogens

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Abstract:

Antibiotic resistance by bacterial pathogens is a persistent problem in medicine. One method of resistance is through RND efflux transporters, which can move antibiotics out of the cells and the periplasm, thus preventing cell death. Many of these transporters can move several very different antibiotics out of the cells, thus conferring multidrug resistance to the pathogens. This project focuses on one such RND transporter found in *Neisseria gonorrhoeae* known as mtrCDE. Our goal is to identify potential inhibitors of mtrCDE by observing their effects on the minimum inhibitory concentrations (MICs) of antibiotics. Ultimately, we aim to develop inhibitors of mtrCDE with the goal of restoring antibiotic sensitivity to antibiotic-resistant *N. gonorrhoeae*. Potential inhibitors have been identified through computational means. In the system described here, the genes for *mtrCDE* have been transformed into an *E. coli* strain that has had the homologous *E. coli* RND transporter deleted for facilitating testing. We have attempted to optimize MIC assays to determine the minimum concentration of antibiotic required to inhibit the growth of cells expressing the *mtrCDE* genes. To date, the largest effects in these assays have been observed with the detergent Triton X-100. In addition, the plasmid encoding *mtrCDE* included a gene conferring chloramphenicol resistance for selection. The media for initial growth of cells contained chloramphenicol to maintain this selection. Initial studies showed that inclusion of chloramphenicol with other antibiotics overwhelms even resistant bacteria. The effects of inclusion of dimethyl sulfoxide (DMSO), a commonly used carrier vehicle for experimental compounds, was tested in the assays since our compound libraries use this solvent. Experiments suggested that inclusion of low concentrations of DMSO does not affect the MIC values observed, but that the cells do reflect a dose response to varying concentrations of DMSO that needs to be carefully controlled. Continuing optimization of these assays with various antibiotics is being performed to determine MIC values, compare them with reported MIC values, and identify antibiotics that are effluxed by mtrCDE that have no published MIC values for *N. gonorrhoeae* or *E. coli*. We report here these and other results of our MIC assay optimization for multidrug resistant, gram-negative bacterial pathogens.

Materials and Methods:

Rapid Colony Transformation: Previously prepared positive-control plasmids were thawed on ice/water, although DNA/protein can quickly be brought to a liquid state if desired. 2.5mL of 50mM $CaCl_2$ were prepared in a 15mL tube and placed on ice/water. Under sterile conditions, a loop was used to pick several colonies of a *BL21(DE3)ΔacrABΔtolC* strain of *E. coli* from a previously prepared LB plate [1X LB supplemented with 15g Agar (Fisher BioReagents) per liter, autoclaved as liquid for 30 min]. The colonies were then transferred to the cold 50mM $CaCl_2$ solution. A sterile pipette tip was primed in chilled sterile water before resuspending the cells. The cells were then split into 0.25mL aliquots in pre-chilled tubes. Next, the DNA was added to the cells (except for a negative control) and swirled to mix, then the samples were incubated on ice/water for 30 minutes. After the incubation, the samples were heat-shocked at 42°C for 2.5 minutes, cooled on ice for 2 minutes, and then moved to room temperature. Next, 0.25mL of 1X LB was added to each sample while at room temperature. The samples were then incubated at 37°C while shaking for 1 hour. Finally, 0.2mL of each culture was plated onto LBC plates [1X LB supplemented with 15g Agar (Fisher BioReagents) per liter, autoclaved as a liquid for 30 min and then 10µg/mL Chloramphenicol was added]. Plates were incubated at 37°C overnight.

Minimum Inhibitory Concentration (MIC) Assays: Cells with either an intact *mtrCDE* coding sequence (positive control plasmid) or the same plasmid carrying double, complete loss-of-function mutations (negative control plasmid) were grown in 10mL LBC (1X LB made to be 10µg/mL Chloramphenicol) at 37°C while shaking overnight. Fresh cultures were made in 10mL LBC using the overnight cultures to start. These new cultures were induced with IPTG at varying times for varying induction periods (testing ongoing for optimization). During growth, a 96-well plate was set up with a serial dilution of antibiotics to be tested (100ul in each well); dilutions were chosen based on expected MIC values reported in literature and any previous experimental results. It is important to note that some antibiotics, such as ampicillin, must be thawed on ice/water. When the induction time being tested was complete, culture was inoculated in 2X LB supplemented with IPTG (made to be 2mM IPTG). The amount of culture inoculated was another variable being optimized and so varied from assay to assay. 0.1mL of culture was added to each well (resulting in 1X LB and 1mM IPTG in the well plate). Plates were incubated at 37°C in a plate reader for 16-20 hours while reading the OD at 600nm. The growth curves were analyzed afterwards to determine the MIC values.

Results:

Selection of a Positive Control with Correct *mtrCDE* Sequence:

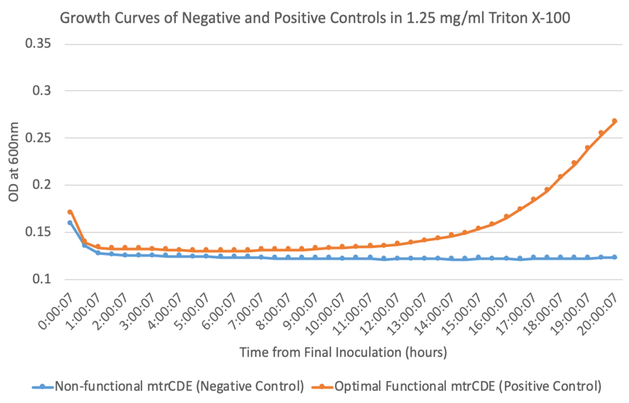

Figure 1: Growth Curves of Negative and Positive Controls in 1.25 mg/mL Triton X-100. This figure demonstrates that the MIC for the positive control must be greater than 1.25 mg/mL Triton X-100, but the MIC for the negative control must be less than or equal to this concentration of Triton X-100.

The positive control in this figure was obtained by rapid colony transformation and was identified to be the strongest positive control of the transformants, giving the greatest signal (difference) against the negative control.

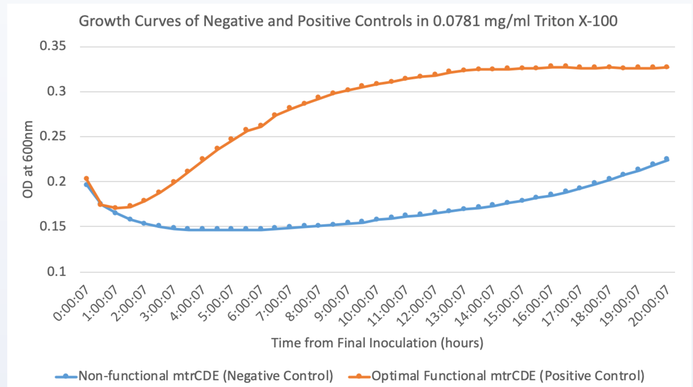

Figure 2: Growth Curves of Negative and Positive Controls in 0.0781 mg/mL Triton X-100. The growth curves in this figure were produced in the same experiment as were those in figure 1. Here, the MIC for the negative control must be greater than 0.0781 mg/mL Triton X-100.

The difference in concentration of Triton X-100 from figure 1 to figure 2 demonstrates the clear signal between positive and negative controls, supporting this positive control as an optimal positive control.

Optimized Cell Concentration for Minimum Inhibitory Concentration Assays:

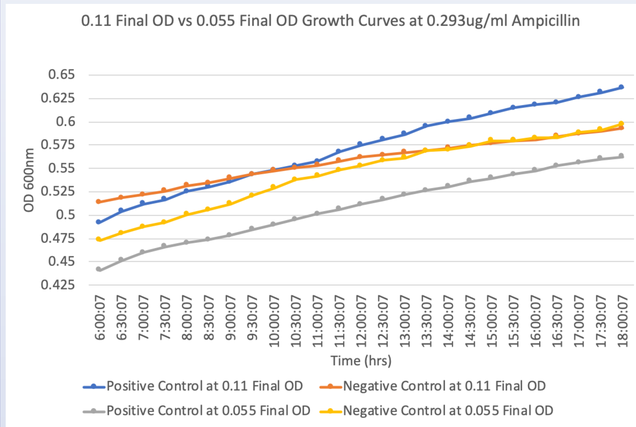

Figure 3: 0.11 Final OD vs 0.055 Final OD Growth Curves at 0.293 ug/mL Ampicillin. While the negative controls ultimately reach nearly identical ODs by the end of the 18-hour read, the 0.11 final OD positive control appears to have a slightly higher rate of growth than the 0.055 final OD positive control. More importantly, the 0.11 final OD positive control outgrew both negative control samples, whereas the 0.055 final OD positive control did not.

This suggests that 0.11 final OD inoculated in the 96-well plate is optimal for MIC assays.

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Identification of a Non-substrate of mtrCDE:

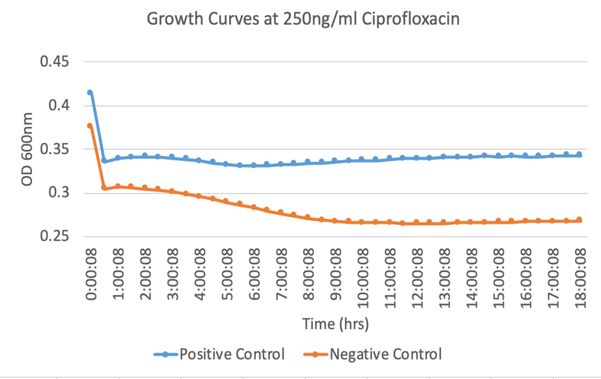
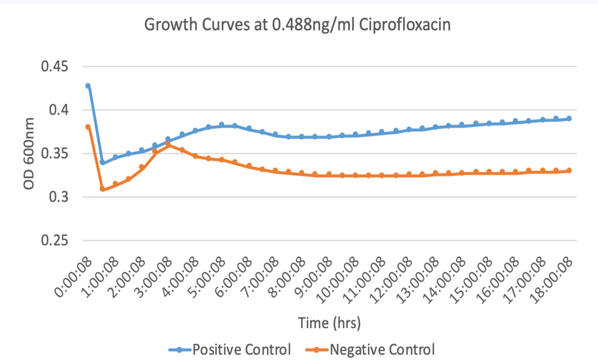


Figure 4: Growth Curves at 250ng/ml Ciprofloxacin. This figure demonstrates that growth of both the positive and negative controls is largely inhibited at this concentration of Ciprofloxacin.

Figure 5: Growth Curves at 0.488ng/ml Ciprofloxacin. This figure demonstrates that both the positive and negative controls tend to grow slowly at this concentration of Ciprofloxacin.



There is no significant observable difference in the growth patterns and MICs of the positive and negative controls at any concentration tested. This indicates that the positive control is no better suited to withstand Ciprofloxacin than the negative control, suggesting that Ciprofloxacin is not a substrate of mtrCDE.

Conclusions:

- **An optimal positive control has been identified**
- **A final OD of 0.11 in the wells of a 96-well plate is an optimal concentration of cells for MIC assays**
- **Ciprofloxacin is not a substrate of the mtrCDE RND pump from *Neisseria gonorrhoeae***

Future Directions:

- **IPTG induction time needs further optimization**
- **Identification of an optimal negative control**
- **Compare reported MIC values in literature to those obtained through experimentation**
- **Test computationally-designed drug-like molecules to identify inhibitors of mtrCDE**

References:

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