The Role of Proteases in Persister Formation in Mycobacterium tuberculosis
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Abstract
More people are killed each year by Mycobacterium tuberculosis (MTb) than any other bacterial pathogen, due to the long and often ineffective treatment regimens required to eradicate the infection. The recalcitrance of chronic MTb infections may be due to persisters, dormant cells that are tolerant to antibiotics. Previous research has shown that toxin-antitoxin (TA) modules present in many pathogenic bacteria are involved in persister formation, but MTb’s possession of over 65 TA systems makes a screen of these operons unwieldy and unlikely to produce useful data. Since the successful action of a toxin depends first on the degradation of its cognate antitoxin, we therefore examined the role of the proteases ClpCP and ClpXP on persister formation. We hypothesized that proteases contribute to persistence in MTb by degrading antitoxin and inducing a state of drug-tolerant dormancy. We began by over-expressing the proteases using an anhydrotetracycline (ATC)-inducible plasmid and measuring the change in antibiotic tolerance. We also knocked down protease genes using ATC-induced transcriptional repression and measured the effect on survival to antibiotics. We found that over-expression of individual Clp subunits did not affect survival to mass action, a fluoroquinolone antibiotic; however, knockdown of the same protease produced 10 fold fewer persisters. These data show that a fully functional Clp complex is required for survival to antibiotics, and identify Clp as a possible target for new drugs targeting non-growing pathogens.

Introduction
Mycobacterium tuberculosis (MTb) kills more people each year than any other bacterial pathogen, despite the availability of drugs that rapidly kill the bacterium in vitro[1]. The current “short course” of treatment, which lasts 6 months for drug susceptible MTb, is necessary to prevent the action of an antibiotic; rather, they stop growing. Since many antibiotics target growing cells, persisters avoid their effects because they do not present any active targets. Toxin-antitoxin (TA) modules[2], such as hipAB and rrsA[3], have been shown to induce dormancy and cause persisters in Escherichia coli, and over-expression of rrsA was shown in MTb to increase multi-drug tolerance[4]. It is likely that other TA modules affect persister formation, but MTb contains at least 65 TA systems[5]. Given the redundancy between persister formation mechanisms[6], screening these 65 TA modules would be unlikely to produce meaningful data.

Results

![Graph showing the relationship of toxin/antitoxin systems and the ClpP/CipP proteases.](image)

Figure 1: The relationship of toxin/antitoxin systems and the ClpP/CipP proteases. A) Under normal conditions, antitoxin binds to and inhibits its cognate toxin. When the antitoxin is degraded, the toxin is free to bind to its cellular targets. B) Activator, which includes the genes encoding proteases, genes both the ClpP protease and a proteasome, indicating that protease expression is likely an important regulator of cellular stress.

Discussion
pTet-Kn-GW can be used to over-express protein in mycobacteria
• Plasmid can be constructed in E. coli and used for protein expression in MTb.
• Reduces time to construct mutant strains since E. coli grows much faster than M. tuberculosis.
• 48 hours induction is required to obtain maximum protein expression in MTb (Fig 6).

Over-expression of protease genes may not affect persister formation in M. tuberculosis
• Fold change in persister level at day 14 was close to 1 for most genes tested. ClpS was an exception at 1.68-fold more persisters, but the results were not statistically significant (p=0.38).

Deletion of protease genes has a greater effect on persister formation in M. tuberculosis
• Fold change in persister level at day 14 was 10.94 for the ClpC1 knockdown strain (p<0.05).

Why does knockdown affect persister formation while over-expression does not?
• The ClpC/ClpP proteases act as a complex, requiring both a functional protease (ClpP) and AAA+ ATPase (ClpC or ClpX) to degrade proteins.
• Over-expression of any one component would not result in a higher concentration of protease complexes, but removal of a component would result in fewer functional protease complexes.

Future Work
• We do not yet know the mechanism by which proteases contribute to antibiotic tolerance. Possible mechanisms include:
  • Degradation of antitoxin, resulting in free toxin and a dormant state
  • Degradation of antibiotic targets
  • Cellular turnover

References

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