Stability and location of mutant proteins in bacterial cells

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Introduction and Background

- Novel protein functions can occur when a series of mutations becomes fixed in the genome. These can be beneficial for the organism, but more often are deleterious.
- It has been hypothesized that the relative fitness of mutant proteins is primarily determined by three factors: catalytic activity at the active site, stability of overall folding structure, and localization in the cell.
- The goal of this project is to study the effects of different point mutations outside the active site on stability and localization, and the influence of these factors on the evolution of protein function. We used as our model ß-lactamase, a bacterial protein that counteracts ß-lactam antibiotics such as ampicillin, normally localized in the periplasm.

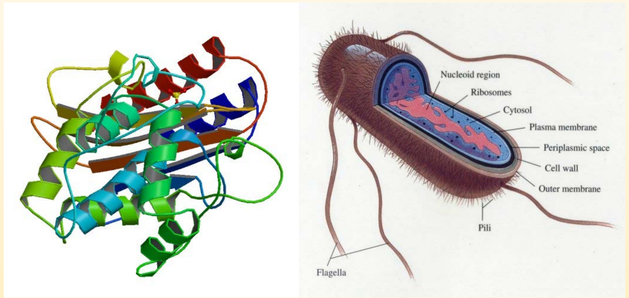

Figure 1. E. coli *TEM-1 ß-lactamase ribbon diagram. PDB: 1BTL. Jelsch, Mourey, Samama 1993.*
Figure 2. *Nemecz, G. "Ultrastructure of an Escherichia coli cell."*

Overall Experimental Plan

- We expressed and purified wild-type and mutant alleles of the chromosomal TEM-1 ß-lactamase gene to study stability and localization of this protein.
- All mutations were outside the protein's active site, removing catalytic activity at the active site as a variable.
- We isolated proteins from periplasmic, cytosol-soluble and insoluble fractions using established biochemical methods.
- Subsequently these protein samples have been or will be analyzed using transverse urea gradient gel electrophoresis and Western blot to determine stability of these alleles, and using ELISA to quantitatively determine localization.

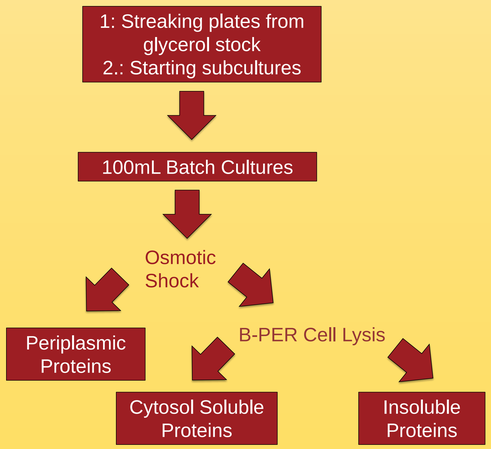

Figure 3. Flow diagram for protein expression and purification.

Materials & Methods

- Used 39 TEM-1 ß-lactamase mutant XL-10 Gold *E. coli* clones assembled by Dr. Scott Wylie
- XL-EP: positive control with single copy of TEM-1 gene on chromosome.
- tev-his pBAD: positive control with multiple copies of TEM-1 gene on plasmids. Designed to express very high ß-lactamase yield.
- Grew 100mL batch cultures of 6 clones with 50µg/mL ampicillin, 39 clones with 15µg/mL tetracycline.
- Used Nanodrop 2000 spectrophotometer to measure $OD_{600}$to determine concentration of cells in culture. Use this to develop rough growth rate curves (see fig. 7-8) for different mutants.
- **Ampicillin** is a ß-lactam antibitiotic that targets bacterial cell walls and is acted on by ß-lactamase.
- **Tetracycline** is an antibiotic that targets protein translation at bacterial ribosomes and is not acted on by ß-lactamase.
- XL-10 Gold cells are ampicillin and tetracycline resistant. Cultures are inoculated with antibiotics to select for experimental cells and prevent contamination.

- Used **osmotic shock** with 20% sucrose Tris-Cl and 5mM $MgSO_4$ buffers to purify periplasmic protein fraction.
- **B-PER cell lysis**: used B-PER reagent to lyse cells and separate soluble cytosolic proteins from insoluble proteins.
- **Concentration using centrifugal filters:** concentrated periplasmic protein samples for easier detection on Western blot.
- **Qubit protein concentration assay:** quantified concentration of protein samples using Qubit protein-specific fluorescent dye and Qubit fluorometer.
- **SDS-PAGE denaturing gel:** gel electrophoresis in gel which denatures proteins to shapeless polypeptide chain, sorting by size of chain.
- N**ative gel:** gel electrophoresis that preserves normal (native) tertiary structure of protein, sorting by size, shape and charge; used to compare with positive control.
- Gels stained with **Coomassie Brilliant Blue** dye (clearer image) and **silver stain** (more sensitive).
- **Western blot:** protein gel electrophoresis results blotted onto PVDF membrane and incubated in anti-ß-lactamase antibody (followed by signal-amplifying secondary antibody) to determine presence of ß-lactamase in mixture of proteins in a sample.

Preliminary Results

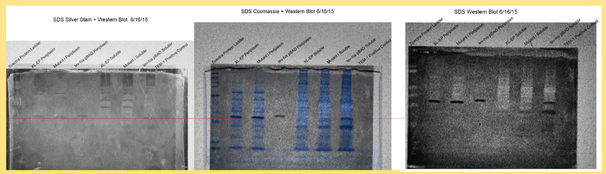

Figure 4(a,b,c). Results of SDS-PAGE gel electrophoresis for XL-EP, tev-his pBAD and mutant i (previously established to have relatively low ampicillin resistance compared to positive controls) as observed by sliver staining, Coomassie Brilliant Blue (Staining) and by western blot.

(a) Western blot overlaid on silver stained gel image,

(b) Western blot image overlaid on Coomassie stained gel image,

(c) Western blot: ß-lactamase signal seen in concentrated periplasmic fraction, absent in soluble fraction.

Preliminary Results

TEM-1 Mutant	Wet Weight of Harvested Cells from Amp-Selected Cultures (g)	Wet Weight of Harvested Cells from Tet-Selected Cultures (g)
0	0.94	0.5
1	1.16	0.38
2	1.16	0.35
4	1.11	0.3
5	1.18	0.49

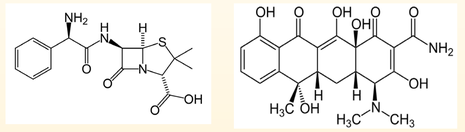

Figure 5,6. Ampicillin (left) and tetracycline (right). *Source: Wikimedia Commons*

- Ampicillin selection marker observed to produce higher total cell yield compared to tetracycline selection. Potential unintended artificial selection for higher ß-lactamase fitness than would be found in native state.
- Tetracycline used as selection marker for experiment with all 39 mutants, found significantly lower total cell yields in mutants 0-5 compared to Amp-selected. Unlike ampicillin, tetracycline is unaffected by ß-lactamase, therefore prevents contamination without putting selective pressure for ß-lactamase production.

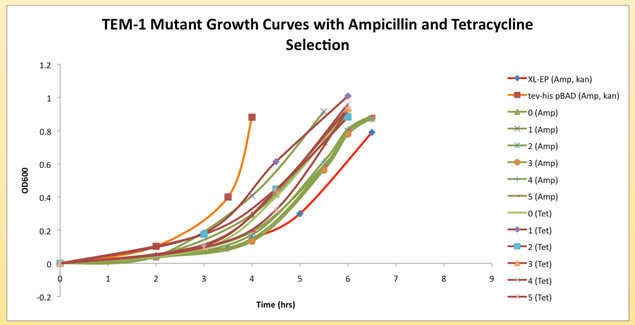

Figure 7. TEM-1 Mutant Growth Curves with ampicillin and tetracycline selection. Choice of selection marker, ampicillin vs. tetracycline, did not appear to have significant effect on growth rates in TEM-1 mutants 0-5.

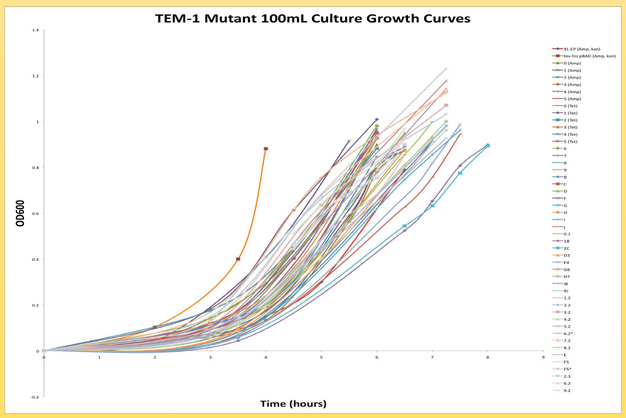

Figure 8 (right). TEM-1 mutant culture growth curves. OD_{600} Growth curves observed to show certain level of variability in rates of growth across all 39 mutants.

Future Directions/Ongoing

- **Transverse urea concentration gradient gel electrophoresis** will be performed on the 39 TEM-1 ß-lactamase mutants, followed by a Western blot. The assay will enable us to assess the impact of mutations on ß-lactamase stability.

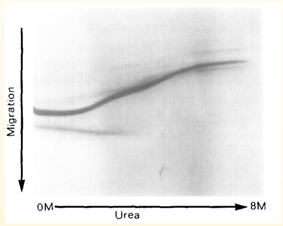

Figure 9. Urea gradient gel electrophoresis of calmodulin. *Goldenberg, Creighton 1984.*

- **ELISA** will be performed to quantify ß-lactamase concentration in mixture of proteins in 39 samples from different cell fractions, which can then be compared to overall protein concentration collected by Qubit assay.

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Acknowledgments

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UTRA Undergraduate Teaching and Research Awards
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