SYNOPSIS OF THE THESIS

Title: Molecular determinants of mutant phenotypes in the CcdAB Toxin-Antitoxin system

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A major challenge in biology is to understand and predict the effect of mutations on protein structure, stability and function. **Chapter 1** provides a general introduction on protein sequence-structure relationships and use of the CcdAB toxin-antitoxin system as a model to study molecular determinants of mutant phenotypes. In **Chapter 2**, we describe the use of saturation mutagenesis combined with deep sequencing to determine phenotypes for 1664 single-site mutants of the *E. coli* cytotoxin, CcdB. We examined multiple expression levels, effects of multiple chaperones and proteases and employed extensive *in vitro* characterization to understand how mutations affect these phenotypes. While general substitution preferences are known, eg polar residues preferred at exposed positions and non-polar ones at buried positions, we show that depth from the surface is important and that there are distinctly different energetic penalties for each specific polar, charged and aromatic amino acid introduced at buried positions. We also show that overexpression of ATP independent chaperones can rescue mutant phenotypes. Other studies have primarily looked at effects of ATP dependent chaperone expression on phenotype, where it is not possible to say whether mutational effects

on folding kinetics or thermodynamic stability are the primary determinant of altered phenotypes, since there is energy input with these chaperones. The data suggest that mutational effects on folding rather than stability determine the *in vivo* phenotype of CcdB mutants. This has important implications for efforts to predict phenotypic effects of mutations and in protein design.

While looking at the mutational landscape of a given gene from an evolutionary perspective, it is important to establish the genotype-phenotype relationships under physiologically relevant conditions. At the molecular level, the relationship between gene sequence and fitness has implications for understanding both evolutionary processes and functional constraints on the encoded proteins. **Chapter 3** describes a methodology to test the fitness of individual CcdB mutants in *E.coli* over several generations by monitoring the rate of plasmid loss. We also propose a methodology for high throughput analysis of a pool of CcdB mutants using deep sequencing to quantitate the relative population of each mutant in a population of *E.coli* cells, grown for several generations and build the fitness landscape.

While the F-plasmid based CcdAB system is known to be involved in plasmid maintenance through post-segregational killing, recent identification of *ccdAB* homologs on the chromosome, including in pathogenic strains of *E.coli* and other bacteria, has led to speculations on their functional role on the chromosome. In **Chapter 4**, we show that both the native ccd operon of the *E.coli* $_{O157}$ strain as well as the ccd operon from the F- plasmid when inserted on the *E.coli* chromosome lead to protection from cell death under multiple antibiotic stress conditions through formation of persisters. Both the ccd_F and ccd_{O157} operons may share common mechanisms for activation under stress conditions and also display weak cross

activation. The chromosomal toxin shows weaker activity as compared to the plasmidic counterpart and is therefore less efficient in causing cell death. This has important implications in generation of potential therapeutics that target these TA systems.

Chapter 5 describes the use of site-saturation mutagenesis coupled with deep sequencing to infer mutational sensitivity for the intrinsically disordered antitoxin, CcdA. The data allows us to make comparisons between overall as well as residue specific mutational sensitivity patterns with that of globular proteins, like CcdB (described in Chapter 2) and study toxin- antitoxin interaction and regulation through saturation suppressor mutagenesis. Interestingly, we found several examples of synonymous point mutations in CcdA that lead to loss of its activity.

In **Chapter 6** we attempt to explore the molecular bases for some of these synonymous mutations. In most cases the mutated codon has a similar overall codon preference to the WT one. Initial findings suggest a change in mRNA structure leading to change in CcdB: CcdA ratio, thereby causing cell death. These observations have important implications, because TA systems are ubiquitous, highly regulated and are known to be involved in multiple functions including drug tolerance. However a role for RNA structure in their regulation has not been shown previously.

Appendix–I lists the mutational sensitivity scores for the CcdB mutants. Phenotypes for CcdA mutants obtained through deep sequencing have been tabulated in **Appendix-II**.

Overall, we provide extensive datasets for mutational sensitivities of a globular (CcdB) and an intrinsically disordered protein (CcdA). Exploration of the molecular

determinants of these mutant phenotypes not only provides interesting insights into CcdAB operon function but is also useful in understanding various aspects of protein stability, folding and activity as well as regulation of gene expression in bacteria.