PROGRAMMED CELL DEATH IN PROKARYOTES: AN INSIGHT INTO THE TOXIN-ANTITOXIN MODULE OF Bacillus anthracis

SUMMARY
The main findings of the present study are:

1. The present study identified a chromosome encoded Toxin-Antitoxin locus (pemlK) from *Bacillus anthracis* (Sterne 34F2). DNA sequence analysis of *B. anthracis* revealed that its genome contains a 351 bp ORF encoding a protein of 116 amino acids, PemK and a 288 bp ORF encoding a 95 amino acid protein, Peml, upstream to the pemK gene. The two adjacent ORFs are found to be tandemly arranged with a gap of tetranucleotides in the genome of *B. anthracis*. Their operon organization was established.

2. The *pemK* and *peml* were cloned in prokaryotic expression vector, expressed and purified as recombinant proteins in *E. coli* as ≈18 kDa rPemK, ≈14 kDa rPeml and ≈35 kDa Pemi.GST.

3. The ectopic expression of the rPemK in *E. coli* (heterologous host) and *B. anthracis* and *B. subtilis* (homologous host) was detrimental to the growth of cells. However, the coexpression of Peml restored the normal growth rate indicating that the antitoxin was able to neutralize PemK mediated cytotoxicity. Also, the effect of toxin expression was not bacteriocidal but was bacteriostatic.

4. The rate of protein synthesis in *E. coli* cells expressing PemK was shown to be severely impeded with no effect on DNA replication. The rPemK degraded total cellular RNA isolated from both *E. coli* and *B. anthracis* but was unable to degrade any form of DNA suggesting it to be a ribonuclease. Also, the rPeml was able to ameliorate the RNase activity of rPemK in a dose dependent manner with a maximal inhibition when the proteins interacted in a stoichiometry of 1:1. The rPemK was able to bind to ribosomes which confirmed its role in translation attenuation.

5. The rPemK was found to be a pyrimidine (‘C’/’U’) specific ribonuclease. The *Km* of the enzyme for rU and rC substrates was observed to be 5 mM and 7 mM, respectively. The *Vmax* obtained for both the substrates were 200 and 212 μmoles/min/mg.

6. The modeled PemK dimer was superimposed on the experimentally determined structure of YdcE with a rmsd of 0.1 Å indicating a high degree
of structural similarity. The catalytic residues of PemK toxin were predicted based on the model and site directed mutagenesis revealed His59 and Glu78 to be critical for PemK mediated ribonuclease activity and are proposed as the probable catalytic acid-base couple. Q21 and Q79 were found to be responsible in stabilizing the active site and were not directly involved in the reaction mechanism.

The extent of binding of the PemK mutants to the Peml was comparable to the wild type protein indicating that the active site is a small pocket which is strikingly different from the antitoxin (rPeml) binding interface.

The rPeml was found to be both thermo- and protease labile in comparison to the rPemK pointing towards poor conformational and physiological stability of antitoxin. Concordantly, the residues at the N-terminus, C-terminus and in the two intervening regions of Peml, comprising 33% of the total residues, are shown to possess high probability of being disordered.

The Peml and PemK were shown to interact with each other in vivo and in vitro. It was also established that the Peml interacts with PemK in a molar stoichiometry of one to generate a complex that is catalytically inactive.

The rPeml which possess extended/flexible conformation was found to attain some structure upon rPemK binding, shown by CD spectroscopy, ANS binding and acrylamide quenching.

Transcriptional fusions of upstream regions of peml with lacZ revealed existence of promoter encompassing both -10 and -35 motifs spanning 57-86 nucleotides upstream of peml. This was substantiated by primer extension indicating that the peml gene has its own transcription start site 100 nucleotides upstream of its start codon.

The sequence alignment and the structure prediction of the Peml indicated that residues 8-52 form a Ribbon helix-helix motif that might be involved in DNA binding, which was further supported by gel retardation assay.

The rPeml (2 μM) was found to bind to the 250 bp upstream region. Step-wise deletions within this 250 bp total promoter minimized the binding to a 200 bp region from the 5’-end of total promoter suggesting the existence of
promoter like elements before 50 bp from the 3'-terminus of *peml*. Competitive EMSA and super shift assay established the binding specificity.

Although, the rPemK alone was not able to bind to the upstream regulatory regions, the complex of rPemI-rPemK attained a higher binding affinity for the upstream regulatory regions.

The N-terminus of PemI was found to mediate DNA binding while the C-terminus was implicated in PemK binding.

Reporter activity with plasmid harboring β-galactosidase under the transcriptional control of the upstream regions of *peml* unequivocally demonstrates the role of PemI protein as repressor of the operon.

The study also illustrates the significance of this module in stress. The expression of *pemlK* transcript and PemK protein was found to be upregulated under stress conditions.

Synthetic 7-8 amino acid long peptides designed to disrupt the TA interaction were found to be effective in micromolar range *in vitro*. This approach can be harnessed as a potential antibacterial strategy against anthrax in future.