Thesis Title

Programmed cell death in *Mycobacterium*: Study of the role of *parDE* genetic loci of *Mycobacterium tuberculosis* H37Rv in macrophage growth and dormancy

Abstract

Mycobacterium tuberculosis H37Rv escapes host-generated stresses by entering a dormant, persistent state. Activation of bipartite toxin-antitoxin modules is one of the mechanisms known to trigger such a state with low metabolic activity. *M. tuberculosis* harbors a large number of TA systems, mostly located within discernible genomic islands. We have investigated both the *parDE* TA systems of *M. tuberculosis* H37Rv and found that only the *parDE2* locus (but not *parDE1*) encodes functional toxin (ParE2) and antitoxin (ParD2) proteins. The *parDE2* locus was transcriptionally active from growth phase till late stationary phase in *M. tuberculosis*. A functional promoter located upstream of *parD2* GTG start-site was identified by 5´-RACE and *lacZ* reporter assay. The ParD2 protein transcriptionally auto-regulated the *parDE2* promoter by interacting through Arg16 and Ser15 residues located in the N-terminus. We scrutinized the toxicity of ParE2 in *recA1* and *recA*$^{+}$ *E. coli* backgrounds to study the role of SOS response as a survival strategy and examine different phenotypes generated due to ParE2 expression. Ectopic expression of *parE2* affected growth and viability of both the *E. coli* strains to different degrees. Live-dead staining (SG-I/Propidium Iodide) revealed that in a short span of 4h, the toxin killed ~54% of the *recA1* strain compared to ~27% cells of the *recA*$^{+}$ cells. In both cases, the majority of the live cells ~99.99% were viable but non-culturable (VBNCs) and only 0.01% of the population formed colonies under standard culture conditions. The results suggested that though more cells survived the toxic effect in the *recA*$^{+}$ strain, VBNC formation was not solely dependent on SOS regulation. The toxic activity of ParE2, crucially dependent on C-terminal residues Glu98 and Arg102, was neutralized by the antitoxin ParD2, both *in vivo* and *in vitro*. Under *in vitro* conditions, ParE2 inhibited mycobacterial DNA gyrase and interacted with the

GyrB subunit without affecting its ATPase activity. The ParE2 toxicity mediated DNA damage, triggered the activation of SOS response, leading to inhibition of cell division and formation of multi-nucleoid, long filamentous cells. ParE2 expression in *E. coli* resulted in a massive increase in *sulA* and *tisB* transcript levels, and loss of membrane potential in the *recA*$^+$ strain, but and not in the *recA1* (mutant) strain. This indicates the SOS-dependence of these phenotypes. Severe morphological aberrations observed under the electron-microscope in the *parE2*-expressing *recA1 E. coli* strain were largely mitigated when the toxin was expressed in the *recA*$^+$ strain. Cells of the latter strain were extensively filamentous with smooth and intact membranes compared to the mutant *recA1* cells that were wrinkled and corrugated with hyper-hydrated periplasmic spaces and large electron-lucent 'vacuoles.' This is an indication that a functional SOS- response is involved in the recovery of ParE2-intoxicated *E. coli* cells. As the effect of the toxin waned with time (perhaps due to proteolytic turnover), the *E. coli* cells resumed division and recovered their colony-forming ability. The ParE2-expressing *recA*$^+$ *E. coli* strain produced significantly higher number of persisters than the *recA1* strain, upon exposure to different antibiotics. Introduction of the *parE2* gene alone into *M. smegmatis* (a surrogate host for Mtb) did not result in any transformants despite repeated attempts. However, an *M. smegmatis* strain containing the complete *parDE2* operon also switched to a non-culturable phenotype in response to oxidative stress. This loss in colony-forming ability of a major proportion of the ParE2-expressing cells suggests its potential role in dormancy, a cellular strategy for adaptation to environmental stresses. ParE2- triggered VBNC formation and persistence are hallmarks of dormancy, potentially relevant in tuberculosis. Our study has thus laid the foundation for future investigations to explore the physiological significance of *parDE2* operon in Mtb persistence and dormancy.