SUMMARY

Pseudomonas aeruginosa causes severe infections in individuals which have compromised immunity due to HIV infections or chemotherapy in addition to incidents of burn wounds and contact lens associated corneal keratitis. Infections of *P. aeruginosa* are very difficult to eradicate due to several cell surface associated and extracellular virulence factors that are regulated by global transcriptional regulators and two component systems.

Small regulatory RNAs (sRNAs) have taken centre stage as promising candidates for regulation of bacterial gene expression and are identified as regulators of cell metabolism, antibiotic resistance, quorum sensing, adaptation to stress, pathogenicity, and nutrient uptake etc. sRNAs exert their regulatory effects by affecting transcription elongation, mRNA translation/stability, and modifying the activity of proteins that act as translational repressors.

This work is aimed at unravelling the role of two sRNAs, *viz.* PhrD and P18 in regulation of pathogenicity of *P. aeruginosa* among the 45-50 reported, uncharacterized sRNAs in strain PAO1. These sRNAs were analyzed using *in silico* programs like Target RNA1, RNA Predator and IntaRNA to determine their putative mRNA targets, out of which assayable virulence factors were selected for further studies. RhlR, the quorum sensing regulator of virulence genes was selected as putative target of PhrD, while alkaline protease secretion protein E and protease IV, major virulence factors involved in establishing acute infections; served as the targets of P18.

The sRNAs were overexpressed from the arabinose inducible *pBAD* promoter in the *E. coli-Pseudomonas* shuttle vector pHERD30T and disrupted by insertion of a gentamicin resistance gene by homologous recombination. Two transcripts from the PhrD overexpression strain, one each from the endogenous and the plasmid borne promoter were observed in the northern blots. Aggregate expression of PhrD from both the promoters was sufficient to see an effect on target gene regulation. Chromosomal PhrD was found to express in LB, phosphate limited PPGAS and nitrogen limited MMP media with maximum expression in MMP as compared to the other two. Overexpression of PhrD caused a 6-fold increase in *rhlR* expression while its disruption reduced *rhlR* levels to 0.2-fold as observed in qRT-PCR. Complementation of disruption with *phrD* restored the *RhlR* levels.

A correlation between PhrD and *RhlR* was established by *rhlR::lacZ* reporter fusion assays and transcriptional analysis of *RhlR* in $PhrD^{+}$ and disruption background in LB, phosphate limited and nitrogen limited conditions. *β*-galactosidase activity of *rhlR::lacZ* was 1.5-fold higher in the WT than in *phrD* disruption background in phosphate limited condition while it was 2.5-fold higher in LB. However a 6-9 fold increase in the overall *RhlR* transcripts was observed in qRT-PCR. Comparing the increase observed in qRT-PCR with that of P3-*rhlR::lacZ* fusion explains that the difference in the fold increase is a likely consequence of the modulation of transcripts arising from P4 promoter also. The expression of *rhlR::lacZ* was very high and maximum in nitrogen limited media but with no difference in WT and

disruption strain. This result could be attributed to the involvement of stringent response mediated by ppGpp in the high expression of *rhlR*, alleviating the need of PhrD under N-limited conditions. The sequence specificity of PhrD-*RhlR* interaction was proven by comparing the *β*-galactosidase activity of *rhlR::lacZ* fusion with intact PhrD interaction region on *RhlR* to that of a fusion with a scrambled sequence within the interaction region, in the WT and *phrD* disruption strains. *rhlR::lacZ* fusion bearing a scrambled PhrD interaction region did not show any increase in expression even in the WT strain. This confirmed the positive influence of PhrD on *RhlR* in a sequence specific manner.

The specific involvement and significance of base pairing interaction of PhrD with *RhlR* mRNA at the stretch of 25 nucleotides at the 5′UTR was also seen in the heterologous host *E. coli* in the presence of PhrD overexpression plasmid. *β*-galactosidase activity of *rhlR::lacZ* fusion with intact interaction region was 10-fold higher than that of the scrambled construct. This result also proved that the interaction between PhrD and *RhlR* could be carried out without the assistance of any *Pseudomonas* specific proteins. However, the *Pseudomonas* background facilitated better expression of *rhlR* than in *E. coli*, probably due to better recognition of P3-*rhlR* promoter by *Pseudomonas* sigma factors or involvement of additional *Pseudomonas* proteins.

Genes for rhamnolipid and pyocyanin pigment production are regulated by RhlR. PhrD overexpression led to a 2.5 and 4-fold increase in the production of rhamnolipid biosurfactant and pyocyanin pigment respectively. Disruption of *phrD* reduced their levels to 0.5-fold which were restored to 1.8-fold on complementation. These results corroborated with the *RhlR* levels measured by qRT-PCR.

This work also focussed on characterization of another sRNA P18, the disruption of which reduced the expression of alkaline protease secretion protein E and protease IV to 0.1-fold as compared to the WT. However, overexpression of P18 also down regulated the protease mRNA levels by 0.5-fold, as against the expectation of up regulation in the target gene expression. Disruption of P18 led to an approximately 40% and 60% reduction in the activity of alkaline protease and protease IV respectively when compared to the WT indicating a positive influence of P18 on the expression of above proteases.

LasR is the primary transcriptional regulator of quorum sensing and indirectly influences the expression of proteases in *Pseudomonas. In silico* analysis showed extensive base pairing between LasR mRNA and P18. Therefore, P18 might influence the expression of the studied proteases via LasR.

Overall this study establishes that PhrD and P18 sRNAs regulate the pathogenicity factors and the associated physiology of *P. aeruginosa* PAO1. Comprehension and understanding of the regulatory pathways employed by sRNAs in influencing virulence of *P. aeruginosa* would open up new avenues in therapeutics and help device alternative strategies against this opportunistic pathogen using sRNAs.