INTRODUCTION

Pseudomonas aeruginosa is a gram negative, opportunistic human pathogen. It is frequently involved in nosocomial infections especially in immunosuppresed individuals, cancer patients, severe burn wounds and cystic fibrosis cases [1]. It maintains a large genome, amongst which are encoded a battery of cell associated virulence factors such as Lipopolysaccharide, flagella and Type Three Secretion System, exopolysaccharides like Alginate and secreted factors like exotoxinA, exoenzymeS, rhamnolipid, alkaline protease, and elastase that make it a highly virulent microorganism.

During the last few years an increasing number of non-coding RNAs (ncRNAs) have been described in different pathogenic bacteria such as *Listeria monocytogenes, Staphylococcus aureus, Vibrio cholerae* and *Pseudomonas aeruginosa*[2]. Non-coding RNAs are bacterial small untranslated RNAs typically 50-500 nucleotides in length. They are important regulatory elements which in response to suboptimal or stressful growth conditions help modulate changes in cellular metabolism to improve the probability of cell survival. The mechanism of action of ncRNAs mainly include- protein binding, mimicking the structure of other RNAs and base pairing with other RNAs which can be *cis*-encoded or *trans* encoded[3].Some examples of ncRNAs regulating virulence genes are: RNA III in *Staphylococcus aureus* which represses the synthesis of Rot, a transcriptional repressor of exotoxin genes[4], RybB of *Shigella* regulates *virB* which encodes a major transcriptional factor of virulence genes[5], PhrS of *P. aeruginosa* activates PqsR which is its key quorum sensing regulator[6].

RATIONALE OF THE STUDY

Although the genome of *P. aeruginosa* is much larger as compared to the enteric bacteria, only a few ncRNAs have been observed in it. To date, approximately 43 ncRNAs have been reported in *P.aeruginosa* PAO1 [7,8,9,10] butthe number is steadily increasing with the genetic screens and the RNA seq technology being widely employed. However a very small number of the reported ncRNAs has been functionally characterized. Thus the current study aims at functional characterization of the reported but uncharacterized ncRNAs which would add to the existing knowledge as to how ncRNAs regulate the pathogenicity of an opportunistic human pathogen like *P. aeruginosa.*

OBJECTIVES OF THE STUDY

1. Detailed bioinformatics analysis of the selected ncRNAs such as PhrD, P18, to determine their promoters, terminators, secondary structure and putative mRNA targets
2. Construction of overexpression and disruption strains of the above ncRNAs
3. Analysis of the influence of overexpression and disruption of these ncRNAs on expression of target genes as obtained from bioinformatic analysis
4. Analysis of proteomic differences under modified levels of ncRNAs
5. *In vitro* assays to study ncRNA/ mRNA target interactions

RESULTS

Putative targets for approximately 28 already reported but uncharacterized ncRNAs in *P.aeruginosa* were identified using online programs, RNA predator and Target RNA. ncRNAs, PhrD and P18,were selected for further studies on the basis of pathogenicity genes as their putative targets and also considering the accessibility of the interacting sequence of ncRNA and mRNA in their respective secondary structures. The ncRNAs were cloned and overexpressed in *Escherichia-Pseudomonas* shuttle vector pHERD30T under the constitutive/L-arabinose inducible pBAD promoter in *P.aeruginosa* PAO1. The chromosomal copy of these ncRNAs was disrupted by inserting a gentamicin marker via homologous recombination. The overexpression and disruption of these ncRNAs were confirmed by sequencing and Northern Blot analysis and PCRs respectively.

Functional characterization of PhrD

PhrD is a 74nt long ncRNA flanked by two hypothetical proteins. Two genes, namely DksA and RhlR were chosen as targets for PhrD. DksA is mnemonic of dnaK suppressor and post transcriptional modulator of extracellular virulence factors [11] while RhlR is involved in quorum sensing and transcriptional activation of rhamnosyltransferase and elastase genes [12]. Northern blot analysis indicated overexpression of 120nt long vector borne copy of phrD and an additional band of 195nt that could be attributed to post transcriptional processing. Disruption of chromosomal copy of phrD was further confirmed by northern blot.

Relative expression of PhrD by qRT-PCR was 214 folds in uninduced pHERDphrD and 32 folds in L-arabinose induced pHERDphrD. This indicates that L-arabinose is not able to induce the expression of vector borne phrD. Several folds expression of phrD in the

uninduced sample could be attributed to constitutive expression of pBAD promoter. The expression level of PhrD was 0.14 folds in ΔphrD.

qRT-PCR indicated a 5 and 7 folds increase in the expression of DksA and RhlR in overexpression strain where as disruption of phrD had no effect on the expression of these genes. DksA is a post-transcriptional regulator of virulence factors and RhlR is central to quorum sensing in *Pseudomonas*, which eventually plays a vital role in colonization in the host. ThusPhrD may positively influence the pathogenicity of *P. aeruginosa* via RhlR and DksA.

Functional characterization of P18

P18 is a 100nt long ncRNA flanked by two hypothetical proteins. The targets chosen for P18 were protease IV and Alkaline protease secretion protein E. These are extracellular proteases of *P. aeruginosa* that are involved in corneal keratitis and can cleave several proteins of immune system [13, 14]. Alkaline protease secretion protein E is a cytoplasmic membrane protein that guides the secretion of alkaline protease from the cytoplasm to the periplasmic space.

A relative quantification of expression indicated 590 folds expression in the L- arabinose induced and 21 folds in uninduced strain of P18.

Expression analysis of putative targets of P18 indicated that both the targets are getting downregulated when the expression levels of P18 are altered. Similar results were obtained when the activities of the two enzymes were measured by chromogenic assays. The activity of protease IV and alkaline protease is reduced by50 and 30% respectively under the overexpression of P18. Thus, P18 might be moderating the invasiveness and favouring the chronic infections by *P. aeruginosa*.

P18 mediated regulatory effects were analyzed by 2D-gel analysis of extracellular proteins of the different strains of P18. A few protein spots were found to be differentially expressed. Their identification is underway.

WORK TO BE DONE

1. In vitro assays to analyze ncRNA/ mRNA interaction
2. Proteomic profiling of the different strains of ncRNAs and identification of differentially regulated proteins

REFERENCES

1. Sonnleitner, E., A. Romeo, U. Bla¨si. 2012. Small regulatory RNAs in *Pseudomonas aeruginosa*. RNA Biology. **9:4**, 364-371.
2. Sonnleitner, E., T. Sorger-Domenigg, M.J.Madej, S. Findeiss, J. Hackermu¨ ller, A. Hu¨ ttenhofer, P. F. Stadler, U. Bla¨si and I. Moll. 2008. Detection of small RNAs in *Pseudomonas aeruginosa* by RNomics and structure-based bioinformatic tools. Microbiology. **154**: 3175-3187.
3. Gottesman, S. and G. Storz. 2011. Bacterial small RNA regulators: Versatile roles and rapidly evolving variations. Cold Spring Harb Perspect Biol doi: 10.1101/cshperspect.a003798.
4. Boisset, S., T. Geissmann, E. Huntzinger, P. Fechter, N. Bendridi, M. Possedko, C. Chevalier, A. C. Helfer, Y. Benito, A. Jacquier, C. Gaspin, F. Vandenesch and P. Romby2007. *Staphylococcus aureus* RNAIII coordinately represses the synthesis of virulence factors and the transcription regulator Rot by an antisense mechanism.Genes Dev **21**: 1353–1366.
5. Murphy,E.R., S.M. Payne. 2007. RyhB, an iron-responsive small RNA molecule, regulates *Shigella dysenteriae* virulence. Infect Immun.**75**:3470-3477.
6. Sonnleitner, E., N. Gonzalez, T. Sorger-Domenigg, S. Heeb, A.S. Richter, R. Backofen, P. Williams, A. Huttenhofer, D. Haas, U. Bla¨si. 2011. The small RNA PhrS stimulates synthesis of the *Pseudomonas aeruginosa* quinolone signal. Molecular Microbiology. doi:10.1111/j.1365-2958.2011.07620.x
7. Sonnleitner, E., and D. Haas. 2011. Small RNAs as regulators of primary and secondary metabolism in *Pseudomonas* species. Appl Microbiol Biotechnol.**91**:63–79.
8. Li, K., C. Xu, Y. Jin, Z. Sun, C. Liu, J. Shi, G. Chen, R. Chen, S. Jin, W. Wu. 2013. SuhB is a regulator of multiple virulence genes and essential for pathogenesis of *Pseudomonasaeruginosa*. mBio **4**(6):e00419-13. doi:10.1128/mBio.00419-13.
9. Wenner, N., A. Maes, M. Cotado-Sampayo, K. Lapaouge. 2014. NrsZ: a novel,processed, nitrogen-dependent, small non-coding RNA that regulates *Pseudomonas aeruginosa* virulence. Environmental Microbiology. **16**(4), 1053–1068.
10. Ferrara, S., S. Carloni, R. Fulco, M. Falcone, R. Macchi, G. Bertoni. 2015. Post-transcriptional regulation of the virulence-associated enzyme AlgC by the σ^{22}-dependent small RNA ErsA of *Pseudomonas aeruginosa*. Environmental Microbiology. **17**(1), 199–214.

11. Jude, F., T. Kohler, P. Branny, K. Perron, M. P. Mayer, R. Comte, C. Delden. 2003. Posttranscriptional Control of Quorum-Sensing-Dependent Virulence genes by DksA in *Pseudomonas aeruginosa*. J. Bacteriol. **185**(12): 3558-3566.
12. Medina, G., K. Jua´ rez, R. Dı´az and G. Sobero´n-Cha´ vez. 2003. Transcriptional regulation of Pseudomonas aeruginosa rhlR, encoding a quorum-sensing regulatory protein. Microbiology. **149**: 3073-3081.
13. Engel L. S., J. M. Hill, A. R. Caballero, L. C. Green and R. J. O'Callaghan. 1998. Protease IV, a Unique Extracellular Protease and Virulence Factorfrom *Pseudomonas aeruginosa*. The journal of Biological Chemistry. **273**(27):16792-16797.
14. Laarman, A. J., B. W. Bardoel, M. Ruyken, J. Fernie, F. J. Milder, J. A. G. Strijp and S. H. M. Rooijakkers. 2012. *Pseudomonas aeruginosa* Alkaline Protease Blocks Complement Activation via the Classical and Lectin Pathways. J Immunol.**188**:386-393.