CONCLUDING REMARKS

*Pseudomonas aeruginosa*, a major nosocomial pathogenic bacterium is the causative agent of opportunistic infections in neutropenic and immunocompromised individuals as well as in severe burn victims and also the primary cause of chronic infections in ventilator-assisted and cystic fibrosis patients, ultimately leading to loss of lung function and death in the latter group. The rising incidences of antibiotic resistance in *P. aeruginosa*, as well as its widespread occurrence in hospitals have proven to be a challenge necessitating the search for yet-unexplored antibacterial development targets. While the traditional antibiotics kill or impede bacterial growth, an important emerging strategy to combat pathogens seeks to target bacterial virulence. *Pseudomonas* typically utilizes a type III secretion system (T3SS) to directly inject effector proteins into host cells and cause disease. T3SS are thus, attractive targets for the development of novel anti-virulence drugs as their inactivation would lead to pathogen attenuation or avirulence, followed by clearance of the bacteria by the immune system.

Previous studies report administration of monoclonal antibodies against T3SS structural proteins to counteract the action of various pathogens. In this context, antibodies against the T3SS needle-tip protein PcrV, which controls T3SS effector secretion, was shown to be effective against laboratory strains of *P. aeruginosa* in animal models [Shime *et al.*, 2001; Faure *et al.*, 2003; Imamura *et al.*, 2007; François *et al.*, 2012; Sato & Frank, 2011]. Small molecule inhibitors demonstrating phospholipase activity of ExoU and ADPRT activity of ExoS were identified, and were protected against death in models of *P. aeruginosa* infection [Galle *et al.*, 2008; Reiniger *et al.*, 2007]. Recently, five compounds that inhibit the activity of *P. aeruginosa* T3SS apparatus and secretion of all its effector proteins were identified in a large-scale compound library screen [Aiello *et al.*, 2010]. Similar chemical inhibitors have also been highlighted for other T3SS in other organisms [Duncan *et al.*, 2012]. An alternative strategy is to interfere with the formation or activity of the T3SS translocation pore than to target the effectors because T3SS effector independent functions of the T3SS seem to play a key role in bacterial pathogenesis.

The aim of the present research was to characterize the pathogenic T3SS virulence factors, which contribute to the pathogenesis of *P. aeruginosa* infections. A detailed understanding of
the mechanism of action of these virulence factors is crucial for the study of *P. aeruginosa*-associated infections and the results of this study can serve to develop new anti-infectives that might be less prone to the development of antibiotic resistance and are efficacious with respect to the acute and chronic forms of *Pseudomonas* infection.

Our research highlights the significance of chaperone PcrH in imparting structural support and stability for functioning of translocator protein PopB in the complex. Predominantly, α-helical PopB was found to be a single species oligomer that exists as a “molten globule” in the native state. PcrH interacts with the exposed hydrophobic domains of PopB with high affinity to form PopB-PcrH complex. In the complex form, PopB was however, found to exist both as 1:1 monomeric and oligomeric hetero complex. pH transition studies also points to the strong possibility of PcrH aiding PopB to adopt various structural conformations with respect to the surrounding pH conditions and thus, efficiently form pores on the host cell. At lower pH, PcrH attained structural changes that possibly assist in the release of PopB for its proper functioning.

Binding studies using PopB deletion constructs provided further insights into the interface of PopB-PcrH interaction. The chaperone PcrH was found to primarily bind with the N- and C-terminal residues of PopB. Deletions up to 100 residues from N-terminal end of PopB inhibited the formation of an efficient complex with PcrH. Surprisingly, this hetero-complex existed only in 1:1 monomeric form even after removal of 60 residues from C-terminal end of PopB. The most remarkable phenomena was the structural transition of PopB from molten globule to rigid structure, upon removal of the terminal residues of PopB involved in interaction with PcrH. Finally, an *ab initio* model structure of PopB, docked to its cognate chaperone PcrH, by employing bioinformatics techniques that strongly corroborated the experimental studies, highlighting the importance of PcrH in imparting structural rigidity to PopB.

Studies described herein this thesis also report the first X-ray crystal structure of the effector toxin ExoT with its cognate chaperone SpcS at 2.1 Å resolution. The dimeric heart shaped chaperone structure of SpcS, displays a strong conservation of structures within class I chaperone of T3SS. Next, we determined the critical residues of the chaperone binding and membrane localisation domain of N-terminal fragment of ExoT that are involved in interaction with the dimeric chaperone SpcS. Kinetic analysis by SPR along with *in silico* dynamic studies by MD simulations of the wild type and mutant chaperones delineates the
role of these chaperonic critical residues regulating effective complex formation with the effector toxin, ExoT by a unique distal inter residue communication.

Thus, an attempt to characterize T3SS virulence factors have been well demonstrated in this research work, by the efficient structural and functional characterization of the translocator protein, PopB with its cognate chaperone PcrH and the effector toxin, ExoT with its chaperone SpcS. This study illustrates the importance of chaperones and their complex formation with the T3SS target proteins. Both class I chaperone-SpcS and class II chaperone-PcrH assists their respective target proteins- ExoT and PopB, in carrying out their vital functions through their unique mechanisms. We have used a highly integrated multidisciplinary approach encompassing computational and biophysical/biochemical techniques along with kinetic analyses, mutagenesis, and X-ray crystallography to provide valuable information about a biological system. A detailed mechanistic understanding of targets is also required for drug discovery in order to develop mechanistically tuned high-throughput screens and to provide a rational basis for drug design. Our meticulous findings thus, provide another dimension in understanding the host-pathogen interaction of T3SS and may help in proposing new strategies to combat infection and reduce virulence caused by T3SS of \textit{P. aeruginosa}.

The last few years have seen remarkable progress in the understanding of the structure and function of the T3SS of \textit{P. aeruginosa} and other pathogenic bacteria. However, much remains to be discovered. For example, we still need a better understanding of the relative contributions of the different bacterial T3SS effectors and the T3SS delivery machine itself, and how they cooperate with one another or with other virulence mechanisms in bacterial pathogenesis. This will require the definition of the physiologically relevant host cell targets, as well as the identification of the physiological role of pore formation. Considering the likely possibility that novel T3SS effector proteins will be identified in the near future by using large scale bioinformatics approaches, the time ahead will be as exciting as the last few years. The ultimate goal is to reveal new general principles of host pathogen interactions and to provide new targets and strategies for antimicrobial therapies.

A great proverb as said by Pablo Picasso “Every act of creation is first an act of destruction”. Scientists worldwide work with this motive in understanding the destructive mechanisms of various pathogenic bacteria and to covert them brilliantly and utilise it for constructive purpose of human life. A perfect example is the recent pioneering study of engineering the
Salmonella typhimurium T3SS in achromosomal, non-replicating nanoparticles derived from bacterial minicells. The elegantly engineered system utilizes this bacterial “nanosyringe” delivering heterologous antigens to the class I antigen presentation pathway stimulating immune responses both in vitro and in vivo. This antigen delivery platform offers a novel approach for vaccine development and cellular immunotherapy [Carleton et al., 2013].

Consequently, T3SS inhibitors also have enormous potential as research tools, capable of helping microbiologists explore host-pathogen interactions involving the T3SS. As the rise of antibiotic resistance coincides with a shortage of antibiotic research, unique drugs and methods of treating bacterial infections must be considered. T3SS inhibitors should function not only as research tools but also as novel antibiotics that do not promote the rapid evolution of resistance. The development of these antivirulence compounds is a complex goal that requires the resources of both academia and the pharmaceutical industry but, when achieved, should bear invaluable rewards for worldwide human health.