INTRODUCTION

Even at the end of 20th century, infectious diseases still occupy the foremost rank in overall mortality rates, with more than 18 million deaths annually. At the same time, chronic forms of infections have become substantially more frequent than acute forms. Numerous clinical and bacteriological studies indicate that most severe chronic somatic diseases are derived from prolonged chronic inflammation caused by various infectious agents [Getts & Miller, 2010]. The wide prevalence of chronic infections is largely due to the fact that modern medicine has no effective means to combat them promptly.

**Mechanism strategy of pathogens causing chronic infections**

The host-pathogen interaction is the most complicated and vital step in chronic infections. The key strategy of the microorganism (pathogen) is to suppress or avoid the host’s defense mechanisms and adapt to the internal environment of the host organism. Additionally, it is known that these pathogen carriers (host) often have symptoms as immuno-suppression, various forms of immunological disbalance, immunological tolerance, immunodeficiency, that brings about favourable conditions for the pathogen to survive and consequently lead to further development of the infection.

The pathogenic activities of the microorganisms are determined by a wide group of pathogenic factors or proteins synthesized within the bacterial cell. Based upon the type of secreted pathogenic factors, as well as the structure of secretion apparatus, seven different systems of secretion have been identified till date. A variety of diverse gram-negative pathogens use type III secretion as a conserved and at the same time highly adapted virulence mechanism. Although these pathogens use additional virulence factors, type III secretion is an essential basic virulence determinant. While the mechanism of protein secretion is conserved, the secreted proteins themselves are highly divergent, and the variety of diseases caused by these pathogens in different hosts is reflected by the multitude of type III secreted proteins.

**Type 3 Secretion System (T3SS)- structure and pathogenesis**

T3SS is found in many gram-negative bacteria (*Chlamidiya, Salmonella, Shigella, Vibrio, Photorhabdus, Pseudomonas, Escherichia, Yersinia, Burkholderia, Bordetella, Aeromonas*)
and plant pathogens (*Erwinia*, *Xanthomonas*, *Pseudomonas syringae*, *Ralstonia solancearum*, *Rhizobium*, and *Panotea* [Dean, 2011] and are a common cause of bacterial pathogenesis. Although evolutionary distant from each other, the basic T3SS architecture is found to be similar in all species. Most of the T3SS genes are laid out in operons, located either in bacterial chromosome (also known as pathogenicity islands) or in plasmids.

**Structure:** The key difference between T3SS and other types of secretion systems is the specific structure of the apparatus for transport of pathogenic factors within the cell. This structure is called a “molecular syringe” or injectisome, through which a great variety of pathogenic microorganisms (both endoparasites and ectoparasites) manifests their pathogenic potential [Beeckman & Vanrompay, 2010]. The molecular syringe or injectisome, resembles a bacterial flagella and is composed of more than 20 different proteins, making it one of the most complex secretion systems. The membrane-anchored part known as the base or basal body is composed of several circular rings and serves as a secretion machine for the outer proteins. The base consisting of an ATPase is also responsible for recognition of secreted proteins, as well as supplying energy to the whole system. The extracellular part of T3SS is formed by a needle composed of many units of one single protein [Blocker *et al*., 2001]. The needle measures ~60-80 nm in length and 8 nm in width and diameter of the needle hole is 3 nm. An inner rod connects this needle to the base of T3SS. All these T3SS structural elements are found to be very conserved and similar in most of the pathogenic bacterial strains [Nguyen *et al*., 2000]. A single bacterium can possess hundred T3SS complexes across its membrane.

**Pathogenesis:** Upon contact of T3SS needle of the pathogen with a host cell, T3SS starts secreting pathogenic factors commonly referred to as translocators and effectors. The special proteins called translocators are secreted first that produce a pore or a channel (translocon) in the host membrane, through which the effectors make their way inside the needle towards the host cell. Mutated bacteria that lack translocators are able to secrete proteins but are not able to deliver them into host cells. Some translocators can even serve a dual role of participating in pore formation and after entering into the cell, act as effectors. Interestingly, most folded effector proteins are too large to pass through the needle opening, so most secreted proteins pass through the needle unfolded, a task carried out by ATPase located at the base of the structure [Galan & Wolf Watz, 2006]. The needle complex recognizes the effectors amongst other cytoplasmic proteins by their N-terminal secretion signal (usually within the first 20
amino acids) that does not cleave off, even after secretion. T3SS activation and secretion can be induced by many factors like lowering [Ca\textsuperscript{2+}] in the growth medium or other factors like temperature, pH, osmolarity and oxygen levels.

Besides these, key structural components of T3SS are the family of chaperonins that are essential for proper induction of T3SS secretion. Each chaperone interacts with its specific translocater/effector protein. When a bacterium does not secrete, its effector proteins are bound to chaperones and float in the cytoplasm. When secretion starts, the chaperones detach from the effectors and the latter are secreted and leave the cell [Akeda & Galan, 2005]. Chaperones and their effectors are often encoded by genes that are adjacent to each other. It is important to note analysis of bacterial proteins that were in complex with specific chaperones revealed their structural similarity, although there was no sequence homology between them. Therefore, it could be suggested that the character of interaction between T3SS chaperones and their effectors is universal for all the bacteria [Birtalan et al., 2002; Stebbins & Galan, 2001; Izoré et al., 2011].

Finally, after secretion, T3SS effectors can manipulate host cells in several ways. The most striking effect is promoting the uptake of the bacterium by the host cell. The bacterial effectors typically manipulate the actin polymerization machinery of host cell that is responsible for maintaining the mobility and changes in cell shape and thus use the host cell’s own machinery for pathogenesis [Satchell, 2009]. T3SS effectors have also been shown to tamper with the host’s cell cycle, and are able to induce apoptosis by activating several proapoptotic proteins such as capsases and/or inactivation of certain antioapoptotic factors, such as NF-κB and mitogen-activating protein kinases (MAP_kinases) [Zychlinsky et al., 1994].

Secretion of bacterial pathogenicity proteins by the type III pathway and their injection into the cytosol of animal or plant cells initiates a sophisticated “biochemical cross-talk” (defined by J. E. Gala’n) between pathogen and host. The injected proteins often resemble eukaryotic factors with signal transduction functions and are capable of interfering with eukaryotic signalling pathways. Redirection of cellular signal transduction may result in disarmament of host immune responses or in cytoskeletal reorganization, establishing subcellular niches for bacterial colonization and facilitating a highly adapted pathogenic strategy of “stealth and interdiction” (defined by J. B. Bliska) of host defense communication lines.
Pseudomonas aeruginosa utilises T3SS as a weapon to establish infection

Pseudomonas aeruginosa is a causative agent of many acute and chronic infections including dermatitis, endocarditis, and infections of the urinary tract, eye, ear, bone, joints, and respiratory tract. It is also frequently found in cases of acute pneumonia, especially in patients who are mechanically ventilated, immuno-compromised or have underlying diseases such as HIV, cancer or cystic fibrosis. This gram negative bacterium primarily uses the Type 3 Secretion System (T3SS) to infect host cells and establish virulence. The T3SS architecture is the same as discussed earlier and essentially comprises of the complex macromolecular machinery (injectisomes), secreted proteins (pore-forming translocators and effectors), chaperones and other accessory proteins.

For all T3SS of P. aeruginosa known so far, the translocator operon encodes three translocator proteins PopB, PopD and PcrV, one chaperone PcrH and a regulatory protein PcrG. PopB and PopD are translocator membrane proteins with two and one putative transmembrane helices, respectively and form soluble complexes with a common chaperone PcrH. The oligomeric forms of the proteins and chaperone-complex have been shown to disrupt cholesterol rich membranes within a pH range of 5-7. The translocator protein PcrV, also secreted by T3SS is necessary for translocation but does not seem to be part of the pore. It may instead form a multimeric scaffold at the tip of the T3SS needle and then facilitate the assembly of the PopB-PopD translocation pore in the host cell plasma membrane.

The effector toxin proteins of T3SS present in P. aeruginosa are ExoS, ExoT, ExoU and ExoY. While ExoU and ExoY are involved in host-cell membrane disruption, both ExoS and ExoT are known as the actual virulence determinants due to the presence of bifunctional GTPase-activating (GAP) and ADP-ribosyltransferase (ADPRT) domains, essential for inhibition of bacterial internalization and epithelial cell migration. However, these effector proteins become secretion competent only after they are tethered together with their respective chaperones. SpcS (formerly known as Orf1) is a common class 1 chaperone for both ExoS and ExoT. The chaperone for ExoU is SpcU but no chaperone has yet been identified for ExoY [Galle et. al. 2012a].

Overview of the thesis

To summarize, T3SS is a very complicated mechanism employed by the various gram negative pathogenic bacteria to cause pathogenicity in the host organisms. Experimental
studies [Cornelis, 2006] reveal that bacteria devoid of translocator proteins can secrete effector toxins yet fail to establish virulence in the host cells. The binding of T3SS translocators and effectors to their respective chaperones is also very sensitive to pH changes of the surrounding medium (from bacterial cellular environment to host cell surrounding). In addition, the T3SS chaperones that serve multiple roles to ensure the efficient targeting of protein substrates for secretion are potential targets for innovative antibacterial drug designing.

The present thesis focusses on the biochemical, biophysical and structural characterisation of the T3SS translocators, chaperones and effector chaperone complex so as to develop innovative techniques to combat the host pathogen interaction and reduce virulence of the pathogens.

The interaction of T3SS translocator protein PopB from *Pseudomonas aeruginosa* complexed with the chaperone PcrH apriori within the bacterial cytoplasm is highly susceptible to pH variations. Chapter 3 describes the pH-dependent structural characterization of PopB and PopB-PcrH complex using various biophysical techniques like circular dichroism, fluorescence spectroscopy, analytical ultracentrifugation, dynamic light scattering and isothermal titration calorimetry. In the native state, PopB exists as molten globule and forms complex with PcrH in 1:1 stoichiometry. PopB also undergoes various pH-based structural changes that vary significantly in presence and absence of its chaperone PcrH.

An attempt to study the intricate details of PopB-PcrH interaction by biochemical and computational techniques is presented in Chapter 4. PCR-based deletion mutations corroborated with SPR studies primarily determine the terminal domains of PopB involved in hydrophobic interaction with PcrH. An *ab-initio* model of PopB, docked to its chaperone PcrH highlights the structural aspects of the PopB-PcrH interaction. The chapter further emphasizes residues critical for oligomerisation as well as translocator-chaperone interaction.

Chapter 5 presents the first crystal structure of T3SS effector protein ExoT from *Pseudomonas aeruginosa* bound to class 1 chaperone SpcS. Structural aspects of the ExoT-SpcS interactions are revealed and molecular dynamics studies along with SPR highlights the hot-spot residues needed for maintaining the ExoT-SpcS interaction.