Introduction
1.0 Introduction:

The interaction between host and pathogen is complex and needs careful and detailed study for understanding the mechanism. The pathogenic microorganisms suppress the host defense system and colonize inside them. Such pathogens can only infect host which often have symptoms like immune-suppression, immunological misbalance, and immunodeficiency. Microbial pathogens may be classified into two broad categories ecologically and evolutionarily- 1) some infect the host accidentally and 2) some are pathogenic by nature. The microorganisms which infect host accidentally can survive in different niches accounting for their partial virulence. These are rarely lethal. In contrast to the pathogens that infect host occasionally, the pathogens that co-associate with their host uses escape mechanism and often go unnoticed. Indeed, close examination of these virulent species not only reveals important insight into the mechanisms of pathogenesis but also contributes to the understanding of the inner workings of the host cell. The interaction of host and pathogen is complex and needs careful and detailed study for understanding the mechanism. The pathogenic microorganisms suppress the host defense system and colonize inside them. They use different types of toxins which are delivered to the host and interfere with their normal vital functions.

1.1 An overview of secretion system:

Recent studies in bacterial pathogenesis have allowed us to understand the complexity that lies underneath host-pathogen relationship. Animals, plants and bacterial models serve great purpose for studying this complex mechanism. Genetic analyses of bacterial virulence factors have shown that the presence of specific pathogenic genes. Located in pathogenic islands, these are one of the key factors that account for the pathogenesis. Such genes are transferred through horizontal transfer between bacteria and account for virulence. Bacterial pathogens with host cells are characterized by factors that are secreted into the extracellular matrix. Such secretary proteins exhibit a wide variety of diverse functions that include proteolysis, haemolysis, cytotoxicity and protein phosphorylation and dephosphorylation. They are transported from the pathogens to the host via secretion systems which are of different types. So far seven different types of secretion systems are known- Type I-VII [1, 2, 3, and 4]. Some of them are sec
dependent (Type II and Type V) while others are sec independent (Type I, Type III, Type IV, Type VI and Type VII).

1.1. Type I secretion system:

Type I secretion system is sec independent system where the proteins are transported to the envelope through the inner membrane. The 60 aminoacids at the carboxy terminal encodes a signal sequence that allows the proteins to be secreted and are not subjected to any proteolytic cleavage. Common examples of proteins secreted through this pathway are α-Haemolysin in *E.coli*, Adenylate cyclase in *Bordetella pertussis*, leukotoxin secreted by *Pasteurella haemolytica*, protease secretion system from *Pseudomonas aeruginosa*.

1.1. II Type II secretion System:

Type two secretion system is a sec dependent pathway. The proteins of this system are characterized by the presence of 30 amino acids N-terminal signal sequence which is cleaved by periplasmic proteases during transport. A number of accessory proteins are also required for normal function [5, 6]. Some well known examples are the sec pathway in *E.coli*, pullulanase secretion by *Klebsiella oxytoca* (6) phospholipase C, and other proteins by *Pseudomonas aeruginosa* (7), amylase and protease secretion by *Aeromonas hydrophila* (8) and secretion of polygalacturonase and other proteins by *Xanthomonas campestris* (8).

1.1. III Type III secretion System:

Type three secretions system are composed of more than twenty different proteins encoded by genes arranged in clusters within the genome. This is a sec independent pathway where toxins are delivered to the pathogens on contact. T3SS are composed of different groups of protein- viz the chaperones, the translocators and effectors, the regulatory protein and the apparatus protein. Each of this group of proteins work together to deliver toxins into the host via a macromolecular complex called injectisome. These toxins impair with host defense mechanism and results in different diseases. *Pseudomonas, Yersinia, Aeromonas, Salmonella, Shigella* etc are some common examples of gram negative bacteria that use such system [9].
1.1. IV Type IV secretion System:

The type IV secretion pathways [10] consist of autotransporters which are exported from the cytoplasm through the sec pathway with a proteolytic cleavage at the N-terminal signal peptide. The information required for transport across the outer membrane lies within the secreted protein. The autotransporters form a pore in the outer membrane through which they are translocated and are released in the supernatant via autoproteolytic cleavage. Vacuolating cytotoxin of *Helicobacter pylori*, a family of outer membrane proteins in *B. pertussis*, and the secreted proteins SepA and EspC from *S. flexneri* and EPEC are some of the common examples of this secretion system.

1.1. V Type V secretion System:

This system uses Sec signals to transport proteins from the bacterial cytosol into the periplasmic space, similar to T2SS; however the translocation of proteins through the outer membrane occurs in the absence of ATP. The type V secretion system (T5SS) is comprised of three comparatively simple systems- the autotransporter (Va), two-partner secretion (Vb) and chaperone/usher pathways (Vc), which allows secretion of proteins through the outer membrane without the input of energy [11]. All T5SS proteins are composed of three domains; an N-terminal signal sequence for transportation through the inner membrane, a passenger domain which will be exposed or secreted into the extracellular milieu, and a translocation unit required for the formation of a pore in the outer membrane [12]. Many bacteria such as *Bordatella pertussis* as well as *Escherichia coli* use monomeric autotransporters, one of the first studied T5SS [13].

1.1. VI Type VI secretion System:

Only recently discovered, the Type VI Secretion System (T6SS) was shown by John Mekalanos to be used by two bacterial pathogens, *Vibrio cholerae* and *Pseudomonas aeruginosa* [14, 15]. This system is thought to act like an inverted phage tail, which ejects effector proteins from the membrane, and is encoded by at least a quarter of all known proteobacteria species [16]. The T6SS is regulated largely through sensor kinase and quorum sensing pathways [17]. Though the
role of T6SS needs to be investigated further but it apparently appears to be a defence mechanism against other bacteria.

1.1. VII Type VII secretion System

Recently in *Mycobacterium tuberculosis* a few proteins with N-terminal Pro and Glu or Pro-Pro-Glu are found to be expressed during virulence. These are term PE and PPE respectively. The PE and PPE proteins interact with each other. These proteins are classified under early secreted antigenic target secretion system (ESX) or commonly called the Type VII Secretion (18, 19). They vary between different organisms and are composed of the following: 1) (i) one or more small helical proteins of the WXG100 protein family (e.g., ESAT-6, YukE), (ii) an FtsK/SpoIII-type ATPase that is thought to drive protein secretion (e.g., EccC, YukB), and (iii) a multipass transmembrane protein that may form the pore of the translocon (e.g., EccD, YueB). *M. bovis* has five different ESX systems (ESX I-V). Besides Mycobacterium, other gram positive bacteria like *Staphylococcus aureus, Bacillus subtilis* also employ Type VII secretion system to impair their host defenses.

![Fig 1.1 Secretion system in bacterial systems: an overview](image)
1.2 Type three secretion system – An insight into the assembly:

Gram negative bacteria employ specialized structures called injectisome for delivery of toxins into host cells using type three secretion systems. It is essential for many clinically relevant bacteria like Pseudomonas, Yersinia, Salmonella, Shigella, Enteropathogenic E.coli (EPEC) etc. They are host specific - Yersinia spp., Salmonella spp., Chlamydia spp., Shigella spp, Pseudomonas aeruginosa, enteropathogenic Escherichia coli (EPEC), Bordetella spp., Burkholderia spp., Citrobacter rodentium and Chromobacterium violaceum [20; 21, 22 ] are mammalian specific pathogens. Fish pathogens like Aeromonas salmonicida subsp. salmonicida, Aeromonas hydrophila, Vibrio parahaemolyticus and Yersinia ruckeri also harbour T3SSs [23,24,25,26] that may be important for virulence [26,23]. T3SSs are also used by plant pathogens, such as Xanthomonas spp. Erwinia spp., Pseudomonas spp. and Ralstonia solanacearum [21]. Made up of >20 different proteins the syringe like assembly spans the bacterial inner and outer membrane into the extracellular matrix. Different structural biological approaches like EM, NMR, crystallography and molecular modeling have been used so far to investigate this assembly. The T3SS assembly consists of (1) chaperones (2) basal body, (3) inner membrane export apparatus, (4) needle and (5) the translocon.

- Chaperones:

  Chaperones are important in the transportation of translocators and effectors inside host cells. Depending on partners they recognize, the chaperones are classified into three broad groups- Class I chaperones which recognizes one (IA) or more effector (IB) molecule(s). Class II chaperones interact with translocators, and Class III chaperones sequester the needle-forming proteins, impairing self-polymerization [27, 28]. Class IA and IB chaperones share a common overall heart-shaped structure, whereas class II and class III chaperones display TPR-like folds. ExsE, an effector involved in transcriptional activation in P. aeruginosa, and its cognate chaperone ExsC also reveals that the latter carries a class IA fold [29] PcrH (P. aeruginosa) and IpgC (S.flexneri) are some of the well known examples of type II chaperones. PscG (P.aeruginosa) and YscG (Yersinia enterocolitica) are classified as type III chaperones. Class II and III chaperones bind early binding substrates (needle proteins and translocators) while class I chaperone binds late binding substrates (effectors and toxins).
• Basal Body:

The basal body traverses the bacterial inner and outer membranes and is made up largely of three different proteins- into a series of highly oligomerized, concentric rings: two proteins localizing to the inner-membrane — PrgK/YscJ/MxiJ (Salmonella, Yersinia and Shigella nomenclature) and PrgH/YscD/MxiG — and the third — InvG/YscC/MxiD — a member of the secretin family of outer-membrane proteins. With the secretins are specialized proteins called proteins, Shigella pilot MxiM [30] is one of the examples of this type proteins. Pilots bind to the C-terminal helix of hydrophobic pocket of secretins.

• The inner membrane export apparatus:

The inner membrane apparatus consists of 5 proteins in T3SS and 6 proteins in the flagellar system. EM analysis showed that the membrane components are predicted to be located in a specialized patch of the membrane at the center of the inner-membrane rings of the basal body [31]. These proteins form an export channel with the cytoplasmic domain of SpaS/YscU/Spa40 and InvA/YscV/MxiA which is the gateway to the channel. The ATPase export complex is thought to localize to the base of the injectisome where the energy for ATP hydrolysis probably uncouples the chaperone-translocator complex and drives the translocator and effector through the injectisome.

• The needle

The injectisome is a long, extracellular protuberance that is built by the helical polymerization of numerous small protein of PrgI/YscF/MxiH family. The proteins consist of helix coiled coil bundle motif linked by PxxP turns as inferred from the pseudo atomic model of MxiH [32]. The inner rod protein like PrgI/YscI/MxiI forms a continuation of the needle by connecting to the basal body and is essential in translocation of the toxic effectors.

• The translocon:

The translocon is a proteinaceous pore forming complex that inserts itself directly into the host cellular membrane. Two hydrophobic membrane proteins —translocators form the pore while the third forms the tip connecting the distal end of the membrane spanning translocon. These hydrophilic proteins proposed to act as adapters linking the injectisome to the host cell membrane. Structural diversity is present in these types of proteins.
However, they consist of a coiled coil topology but vary in terminal portions accounting for their different functions. Examples of such proteins are LcrV (*Yersinia*), EspA (*E.coli*), BipD (*Burkholderia*) and IpaD (*Shigella*) [33, 34, 35, 36, 37, and 38]. Details of this protein assembly are discussed below.

The information regarding the topology of the translocon remains largely unclear due to the lack of structural evidence [39]. These translocators are recognized by their respective chaperones and are channelised through the injectisome in an ATP dependent manner. The translocators have one or two transmembrane region composed of hydrophobic amino acids that allow them to bind to the membranes. The translocators are of two types- 1) hydrophobic and 2) hydrophilic. Hydrophobic translocators are further classified into i) major and ii) minor translocators.

- **Hydrophobic translocators:**
  
  Hydrophobic translocators are recognized by their respective chaperone which consists of TPR motif. They are classified into major translocators i.e. translocators with two transmembrane region and minor translocators i.e. translocators with one transmembrane region.

  i) **Major Hydrophobic translocators:** The major translocators consist of two transmembrane regions, an N-terminal coiled-coil region and occasionally a C terminal amphipathic helix. Within the two TM region and intervening loop that the translocators display the highest level of sequence identity demonstrating the requirements of this protein in pore formation. Major hydrophobic translocators are PopB (*P.aeruginosa*), YopB (*Y.enterocolitica*), SipB (*Salmonella sp*) and IpaB (*S.flexneri*).

  ii) **Minor Hydrophobic translocators:** The minor translocators consist of one transmembrane region and have greater sequence identity in comparison. Examples that can be categorized under this group are PopD (*Pseudomonas*), YopD (*Yersinia*), IpaC (*Shigella*) and SipC (*Salmonella*)
- **Hydrophilic translocators:**

Hydrophilic translocators are the third group of translocators that are present in pathogenic bacteria. The hydrophilic translocators are multifunctional macromolecules that play roles in different processes such as regulation of secretion, host process hijacking and toxin translocation; this latter function appears to be the only one that is common to all bacteria. The hydrophilic translocators are recognized by specific small regulators that probably assist them during infection. Common examples of these hydrophilic translocators are the V antigens or LcrV and PcrV in *Yersinia* and *Pseudomonas sp* and the specific regulators are LcrG and PcrG, binding to them in the ratio 1:1 [40 41].

Fig 1.3 T3SS systems – flagellar and non flagellar. a) Flagellar T3SS system b) T3SS in Ysc – yop *Yersinia pestis* [Adapted from Cornelis G (2006) nat rev (Microbio) vol 4 811-825] c) Role of FliI in transport of toxins in *Salmonella sp* [Adapted from Namba K et al. J. Mol. Biol. (2006) 360, 510-519]
1.3 Type three secretion system and its correlation with metabolic pathways:

Pathogens require metabolites for infection. Specific nutrients with other environmental conditions stimulate the production of virulence factors in many pathogens [42]. Stress response, catabolite repression, and carbon storage regulator system play an important role in the control of virulence traits [43; 44; 42; 45, 46]. Type three secretions in gram-negative bacteria is a well studied virulence pathway. The correlation of this pathway with that of metabolic pathway is not well established. In some microorganisms, key metabolites like histidine, aspartate, terathione, glutamate and sRNA play a crucial role in controlling virulence pathways. Among these metabolites, acetyl coenzyme A is an important molecule that links metabolic and virulence pathways. It is produced during the glycolytic pathway and is the main components of TCA cycle, fatty acid biosynthesis, glyoxylate pathways etc. The molecule also controls the Type Three secretion system (T3SS). The T3SS is triggered when the pathogenic bacteria comes in contact with host mediated by complex signaling pathways. Based on these findings we have tried to find out the correlation of Coenzyme A biosynthesis pathway and T3SS. Coenzyme A in bacteria is synthesized in five different steps. One of the enzymes-PPAT is important in controlling the CoA biosynthesis as it controls the rate-limiting step of the process. The enzyme is encoded by a gene named coaD. The formation of CoA involves two enzymes one encoded by coaD and the other encoded by coaE. Acetyl CoA is the most prevalent thioester of Coenzyme A in the cell. Intracellular levels of CoA and Acetyl CoA affect the regulation of this enzyme making it an interesting choice of study. Coenzyme A plays a key role in the formation of AcCoA which in-turn regulates the type three secretions. The details of this study have not been well established anywhere till date but will be interesting to know. In one of the thesis chapter we have tried to hypothesize the correlation between T3SS and Coenzyme A pathway in Pseudomonas sp [185]
Fig 1.4 Correlation between T3SS and metabolic pathways in Yersinia. [courtesy Heroven and Dersch frontiers in cellular and infection micro biology 2014 (4): 140: 1-13]

Fig 1.5: Correlation between T3SS 1 & 2 in Salmonella and tetrathionate for colonization in gut. [courtesy Wilharm and Heider Frontiers in Cellular and Infection Microbiology October 2014 Volume 4 Article 150 : p1-10]
2.0 Overview of the thesis:

T3SS is a complex mechanism where gram negative bacteria deliver toxins into the host cell [47]. The delivery of toxins through the injectisome takes place in an energy dependent manner. T3SS ATPases play a significant role in delivery of such proteins. To achieve this they form a hexameric [48] or sometimes dodecameric [48, 49, and 50] assembly near the injectisome. Several T3S ATPases have been partially characterized including EscN from E.coli [49] YscN from Yersinia [51] and InvC from Salmonella [52]. These ATPases show significant similarity with flagellar ATPase FlII in Salmonella. The ATPases have significant sequence orthology with the beta subunit of F0F1 ATPase and the hydrolysis of ATP serves as driving force for releasing the chaperone from cognate effector protein [53]. These ATPases are inhibited in the cytoplasm and tethered to the membrane by specific regulator protein. In *Yersinia enterocolitica*, two different T3SS exists- one is genomic encoded and the other is plasmid encoded pYV system. The genomic encoded- Ysa Ysp system is relatively new and remains uncharacterized. YsaN is the ATPase that translocates protein in the Ysa-ysp system. The enzyme is quite similar to that of YscN ATPase in pYV system which accounts for the same function. Reports in earlier studies suggest that metabolites play a key role in controlling the virulence of T3SS in *Pseudomonas aeruginosa*, *Salmonella*, and *Yersinia pestis*. Among these metabolites, AcCoA is an important molecule in this respect. The major source of synthesis of acetyl-CoA is Coenzyme A and pyruvate. In this respect, the glycolytic pathway and Coenzyme A biosynthesis pathway are important and involved in regulation of T3SS.

In the present thesis, the crosstalk between type three secretion associated proteins and vitamin biosynthesis pathway are characterized structurally biophysically and biochemically in *Yersinia enterocolitica* and *Pseudomonas aeruginosa* respectively. In this respect, YsaN (the pivotal enzyme) along with its regulator (Ye3555) has been identified and studied experimentally in the less characterized Ysa-Ysp T3SS system present in *Yersinia enterocolitica*. Further, Phosphopantetheine adenlyltransferase (PPAT/ coaD), one of the rates limiting enzyme of pantothenate (vitamin B5) and CoA pathway, has been studied in *Pseudomonas aeruginosa*. Acetyl-CoA the primary linking molecule between the aforesaid virulence and metabolic pathways is partially established and indicates metabolic correlation in these organisms due to molecular co-evolution.
In Chapter 1 a detailed description of the earlier studies is mentioned between virulence pathway and metabolic pathway with special references to the correlation between type three secretion system and pantothenate (vitamin B5) and CoA biosynthesis in *Yersinia enterocolitica* and *Pseudomonas aeruginosa* as source organisms respectively.

In Chapter 2 the protocols and methods used in the work are provided with details.

In Chapter 3 YsaN is identified and characterized enzymatically as Mg\(^{2+}\) ion ATPase along with the identification of the regulator protein Ye3555 within the Ysa-Ysp system. Biochemical and biophysical methods were employed to understand the physiological properties of the enzyme and its regulator. The protein has enhanced catalytic activity in the oligomeric state. It is essential for translocation of toxic proteins in the Ysa-Ysp system during pathogenesis and is regulated by YE3555.

In Chapter 4 the allosteric mechanism of Phosphopantetheine adenylyltransferase—the rate limiting step in CoA biosynthesis is described. The most important finding of this enzyme is that it interacts with Acetyl Coenzyme A, which in-turn controls the type three secretion system protein. We have also studied the molecular mechanism underlying this inhibition and compared it with the substrate binding mode. Biophysical and biochemical studies were performed to correlate the structural findings.