Chapter 1

Review of literature
3.0 Review of literature:

3.1 Nomenclature and anatomy of *Yersinia enterocolitica*:

*Yersinia enterocolitica* is a gram-negative bacillus shaped bacterium belonging to the family Enterobacteriaceae. The organism is an agent for mesenteric lymphadenitis. A single colony is 0.5 mm, nonhemolytic convex, smooth, glistering translucent grey with a beaten copper appearance. These microbes biochemically produce acid from xylose, produce iodole and cannot ferment salicin. The organism is susceptible to ampicillin, chloramphenicol, colistin, sulfasoxazole, kanamycin, streptomycin, tetracycline, and gentamycin. *Y. enterocolitica* is a readily identifiable but seldom reported bacterium of clinical significance. It is similar morphologically and biochemically to *Y. pseudotuberculosis* but appears to be antigenically distinct.

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phylum</td>
<td>Proteobacteria</td>
</tr>
<tr>
<td>Class</td>
<td>Gamma proteobacteria</td>
</tr>
<tr>
<td>Order</td>
<td>Enterobacteriales</td>
</tr>
<tr>
<td>Family</td>
<td>Enterobacteriaceae</td>
</tr>
<tr>
<td>Genus</td>
<td>Yersinia</td>
</tr>
<tr>
<td>Species</td>
<td>Y.enterocolitica</td>
</tr>
</tbody>
</table>

3.2 Genome of *Yersinia enterocolitica*:

The genome of *Y. enterocolitica* is very similar in size, the number of predicted genes, and nucleotide composition to those of *Y.pestis* and *Y. pseudotuberculosis*. *Y.enterocolitica*. It contains a similar number of pseudogenes (67CDS) compared to *Y. pseudotuberculosis* (62 CDS) *Y. pestis* contain about 140 CDS as compared to *Y.enterocolitica*. The complete sequence consists of a chromosome of 4,615,899 bp, with a G+C content of 47.27%, and a plasmid (pYVe8081) of 67,721 bp, with a G+C content of 43.92%. The methionine-salvage pathway and osmoregulated periplasmic glucan biosynthesis pathway is completely absent in *Y.pestis* whereas it’s present in *Y.enterocolitica* and *Y. pseudotuberculosis*. *Y.enterocolitica* consist of plasticity
zone ranging from ~2-200kb that contributes to virulence and is responsible for encoding Type three secretion system. Within the plasticity zone lays the niche adaptation genes which is responsible for osmoregulation and osmoprotection and chitinase. The Hyd2 and Hyd4 gene cluster encodes for using local generated hydrogen to co-localize in the gut of host.

3.3 Pathophysiology of *Yersinia enterocolitica*:

*Y. enterocolitica* cause gastrointestinal symptoms ranging from self limiting diarrhea to mesenteric lymphadenitis sometimes leading to appendicitis [54, 55]. The severity of infection is dependent on the serotype and biotype of the organism. Gastroenteritis is one of the major symptoms that affect infants and young children under 5 years [55]. In older children one of the most common symptoms due to infection caused by yersinia is yersiniosis. Sepsis due to Yersinia takes place during blood transfusion [56]. *Yersinia* has over 11 species of which *Yersinia enterocolitica, Yersinia pseudotuberculosis* and *Yersinia pestis* are pathogenic to rodents and human and causes diseases ranging from plague to gastroenteritis. Based on biochemical features *Y. enterocolitica* is divided in 6 biogroups, namely 1A (non pathogenic), 1B (highly pathogenic) and bio groups 2, 3, 4 and 5 that are weakly pathogenic. On a worldwide basis the majority of these infections are caused by serotypes O: 3, O: 9, O: 5, 27 and O: 8 [57]. The bacteria cross the intestinal barrier in the Peyer’s patch of ileum [58]. Invasin is the outer membrane protein that binds with b1 integrins, expressed atypically on M cells. Surface protein like Ail, PsaA and YadA may account for residual invasion in yersinia sp [59]. Once the dome is reached the yersinia survive attack by resident macrophages and encode antiphagocytotic proteins using T3SS that disrupts the cytoskeletal assembly of host protein [60].

![Fig3.1 Physiological infection of Yersinia](adapted from P. Sansonetti 2002, Gut, vol. 50, no. 3, pp. iii2–iii8)
3.4 Type Three Secretion of *Yersinia enterocolitica*:

*Yersinia enterocolitica* is a gram negative bacterium classified into different biovar based on biochemical properties. Among these the biovar 1B strain contains a specific region of 200kb that is not found in other *Yersinia* strains [61]. This is known as the plasticity zone and encodes genes that are essential for virulence. In *Y.enterocolitica* 8081 1B, apart from the Ysc-Yop (yersinia secretory chaperone-yersinia outer proteins) T3SS, there lies an additional T3SS called the Ysa-Ysp (Yersinia secretory apparatus- Yersinia secretory proteins) system. The two secretion system is required for full virulence [62, 63 and 64]. The genes in Ysa locus remains largely obscured and require experimental verification for their functionality and structuralism. However, preliminary work suggests that that the ysa locus consist of a chaperone –SycB, four secreted proteins yspBCDA, some regulatory proteins ysaE, (AraC-Xyls transcription regulators), ysrT (histidine kinase of two component system), ysrR and ysrS (two component system proteins). YsaQ, YsaK, YsaJ, YsaV, YsaT are some orthologues of the Ysc-Yop T3SS that act as the apparatus protein denoted as per electronic annotations. Such system is highly similar Inv/Mxi-Spa family T3SS and has similar genetic organization with Salmonella SPI-1 and Shigella sp [65]. In addition, closely related systems are found in *Sodalis glossinidius*, a plant symbiont [66], and in *Chromobacterium violaceum*, which is found primarily in soil in tropical regions but can cause lethal infections in humans [67].

Almost all T3SS are responsible for delivery of toxin delivery to the host and to attain this they require an ATPase. In the plasmid encoded pYV system the substrates and effectors are translocated via their respective chaperones and YscN acts as the energizer [68]. In the Ysa-Ysp system YsaN functions as the ATPase for translocation of toxins. Several T3S ATPases have been partially characterized including EscN from E.coli [49], YscN from Yersinia [51] and InvC from Salmonella [52]. These ATPases have been shown to have sequence orthology to the β subunit of the F0F1 ATPase and to hydrolyze ATP. The flagellar system FliI ATPase is very similar to that of T3S ATPases and is studied extensively for mechanism of export of toxic proteins. Both the proton motive force and ATP hydrolysis is essential for transport of toxic proteins. ATP hydrolysis is required for translocation of toxic proteins and proton motive force enhances the translocation [69]. The FliI in Salmonella has increased activity in the presence of phospholipids [51]. The ATPase in type three secretions are controlled by specific regulators.
For FliI (ATPase) its FliH (regulator) [69], YscN (ATPase) is controlled by YscL (regulator) [51]. In case of YsaN ATPase, the regulator protein was not known earlier. YE3555 was analyzed using bioinformatics tool and validated experimentally as the negative regulator of YsaN [50]. This has been discussed in details in Chapter 3. It is mentionworthy that the T3S ATPases requires such additional regulators for tethering into membranes for proper functionality. Such regulators are essential in the biological mechanism involved in this complex system and are worthy of study.

3.5 Nomenclature and anatomy of *Pseudomonas aeruginosa*:

*Pseudomonas aeruginosa* is a gram negative, rod shaped asporegenous, and non-flagellated bacterium with increasing nutritional versatility. Scientific classification is as follows:

<table>
<thead>
<tr>
<th>Kingdom</th>
<th>Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phylum</td>
<td>Proteobacteria</td>
</tr>
<tr>
<td>Class</td>
<td>γ Proteobacteria</td>
</tr>
<tr>
<td>Order</td>
<td>Pseudomonadales</td>
</tr>
<tr>
<td>Family</td>
<td>Pseudomonadaceae</td>
</tr>
<tr>
<td>Genus</td>
<td>Pseudomonas</td>
</tr>
<tr>
<td>Species</td>
<td>aeruginosa</td>
</tr>
</tbody>
</table>

*P. aeruginosa* resembles a rod measuring about 1-5 µm long and 0.5-1.0 µm wide. It is an obligate respire, using both aerobic respiration (with oxygen) as its optimal metabolism or anaerobically on nitrate or other alternative electron acceptors. This organism can catabolize a wide range of organic molecules, including organic compounds such as benzoate and hence is a very ubiquitous microorganism, found in soil, water, humans, animals, plants, sewage, and hospitals [70]. In all oligotrophic aquatic ecosystems, which contain high-dissolved oxygen content but low plant nutrients, *P. aeruginosa* is the predominant inhabitant and this clearly makes it the most abundant organism on earth [71].
3.6 Genome of *Pseudomonas aeruginosa*:

As of September 2013, 36 strains of *P. aeruginosa* from both clinical and environmental sources had been fully or partly sequenced according to the NCBI Entrez database. Compared with the other disease causing bacteria, *Pseudomonas* has a relatively large genome, ranging from 6.22-6.91 mega basepair [72] with 65% (G+C) content. Sequencing of these multiple strains reveals the genome as an assortment of conserved regions interspersed by ‘regions of genomic plasticity’ that contain genes unique to each strain [73]. The variable accessory genome is characterized by a set of genomic islands and islets from a primeval tRNA-integrated island type. The core genome consists of 0.5% nucleotide divergence and a conserved synteny of genes, which means two or more genes, whether they are linked or not, are on the same chromosome [73]. *P. aeruginosa* also possesses a single and supercoiled circular chromosome in the cytoplasm [74], along with chromosome-mobilizing plasmids that are vital to the pathogen. For example, plasmids TEM, OXA, and PSE that encode betalactamase production are essential for antibiotic resistance [75].

3.7 Pathophysiology of *Pseudomonas aeruginosa*:

*Pseudomonas aeruginosa* is an opportunistic human pathogen, as it rarely infects healthy individuals. Instead, it often colonizes immunocompromised patients, having cystic fibrosis, cancer, or AIDS [77]. This potent pathogen is responsible for attacking upto two thirds of critically-ill hospitalized patients, carries a 40-60% mortality rates at most medical centers, and complicates 90% of cystic fibrosis deaths. It is also listed as one of the top three most frequent gram-negative pathogens and is linked to the worst visual diseases and respiratory infections of hospitalized patients. Airway infections are often classified into two types, acute or chronic, and transmission can be either hospital- or community-acquired, although the latter is rare and almost always associated with an underlying defect in immunity [78]. The high incidence of *P. aeruginosa* in healthcare institutions is contributed to by the poor health status of the patients, high carriage rate of often multidrug-resistant strains in hospital wards, prior use of broad spectrum antibiotics and finally the intrinsic resistance of *Pseudomonas* to several chemotherapeutic agents and antibiotics, making it a very hard pathogen to eliminate [70; 79]. In individuals with CF, a mutation in the cystic fibrosis transmembrane regulator (CFTR), a cAMP-
dependent chloride channel, results in a dehydrated and thickened airway surface liquid (ASL) that hinders mucociliary clearance from the conducting airways. Inhaled bacteria take up residence in the altered ASL and cause an initial acute infection and vigorous inflammatory response. The thickened ASL severely impairs the immune response, and the persistent immunological stimulation by the bacteria and/or the inability of the host to control inflammation results in chronic lung inflammation [80; 81]. There are also some additional evidences that the CFTR mutation itself influences the ability of the host to control bacteria-induced inflammation [82]. The destruction of lung function due to the hyperactive inflammatory response, possibly exacerbated by bacterial toxins, causes the progressive deterioration of lung function and ultimately makes these lung infections fatal.

3.8 Vitamin biosynthesis and pantothenate CoA pathway in Pseudomonas aeruginosa:

Enzymes require co factors and coenzymes for catalytic activity. These cofactors are synthesized from vitamins. Vitamins are nutrients which are required in small amounts to ensure normal vital functions of living cell. Pantothenate or vitamin B5 is one several vitamins that is required for synthesis of coenzyme A - a vital cofactor used in different pathways from TCA cycle to fatty acid biosynthesis. The phosphopantetheine moiety is transferred to acyl carrier protein for poly ketide synthesis and nonribosomal protein synthesis [139 140 141 & 175]. CoA is produced from pantothenate in 5 different steps and is studied in a variety of organisms. The first step is an ATP dependent phosphorylation to form phosphopantothenate catalysed by pantothenate kinase (PK, panK/coaA). Subsequent ATP-dependent ligation of cysteine by phosphopantetheine synthetase (PPCS, coaB) to give and flavin independent decarboxylation by phosphopantotheine decarboxylase (PPCDC, coaC) yields phosphopantetheine. This is subsequently adenyLATED by phosphopantetheinyladenyl transferase (PPAT, coaD) to form dephosphoCoenzyme A which is then phosphorylated to form coenzyme dephosphoCoenzymeA kinase (dPCK, coaE). The penultimate step catalysed by coaD or Phosphopantethein adenyltransferase is a rate limiting step. It catalyses the reversible conversion of phosphopantetheine to dephosphoCoenzyme A by adenylylation where ATP is converted to pyrophosphate or vice versa. The enzyme is feed back regulated by CoA and is allosteric by nature with overlapping substrate or product and inhibitor (CoA) binding site. Till
now, a number of PPAT X-ray crystal structures have been solved: *viz E. coli* in complex with multiple ligands; [152 &153] *Thermus thermophilus* in complex with PhP [154] *M. tuberculosis* with different ligands [155], *Enterococcus faecalis* [156] and *Helicobacter pylori* [157] and *Staphylococcus aureus* [158] in complex with 3′-phosphoadenosine 5′-phosphosulfate *Burkholderia pseudomallei* [159] in complex with hydrolyzed dPCoA [Protein Data Bank (PDB) ID 3K9W] , and *Yersinia pestis* in complex with CoA (PDB ID 3L92 2010). In most cases the enzyme seemed to form hexamers except for *E.coli* where the enzyme uses Mn$^{2+}$ instead of Mg$^{2+}$ and is dimeric. Unlike the close orthologues, *Pseudomonas aeruginosa* is resistant to pantothenamides. This is experimentally proved by the fact that it is resistant to the uptake of extracellular pantothenate and is not phosphorylated by the type III CoaA PanK. Study of such pathway with differences is important in this organism especially the impact it creates to the enzymes located downstream, required for the conversion of pantothenate to CoA. Since the other rate limiting step down stream to CoaA (panK) is catalysed by CoaD or PPAT, the mechanism involved in this enzyme is study worthy. In chapter 4, a detail description of the biological mechanism for PPAT isolated from *Pseudomonas aeruginosa* has been provided. In this connection, it was found that PPAT binds with a new inhibitor acetyl CoA as per *invitro* studies. Other biophysical and biochemical methods were used to characterize that further. Such binding of molecule indicates correlation between the T3SS and pantothenate pathway.

3.9 Crosstalk between pantothenate pathway and Type three secretion system

The relationship between virulence and metabolic pathways are highly complex and difficult to understand [83]. Among different virulence pathways one of the important examples is type three secretions which have been used to study and correlate different metabolic pathways. Among the metabolic pathways pantothenate and coenzyme A biosynthesis is important vitamin biosynthesis pathway that shows direct control of type three secretion sytem via acetyl CoA first reported in *Pseudomonas* in erlier studies [88]. In *Yersinia* [84, 85, 86] and *Salmonella* [87] such studies indicating correlation between virulence and metabolic pathway has been reported too. The correlation between type three secretion and metabolic pathway is described further in details for *Yersinia enterocolitica* and *Pseudomonas aeruginosa*.
**Yersinia enterocolitica:**

**Ysa-ysp system:**

*Yersinia enterocolitica* has well characterised plasmid encoded Ysc-Yop (*Yersinia* secretory chaperone-*Yersinia* outer membrane proteins) T3SS. But recently, it was reported that in addition to this 70kb pYV encoded Ysc-yop T3SS, a genomic encoded T3SS called the Ysa-Ysp (*Yersinia* secretory apparatus-*Yersinia* secretory proteins) exists. The ysa pathogenicity island encodes for 18 genes, organized as single transcriptional unit upstream of promoter ysaE, to constitute the YsaYsp T3SS [89] (Figure 7). The genes encode proteins like regulators, structural components and export apparatus and effectors [89]. The promoter that present upstream of sycB drives the expression of sycByspBCDA operon [89]. Initially it was thought that the organism responds to external stimuli like high salts and calcium ions via two component system with two proteins viz YsrR and YsrS. Later it was found an additional protein YsrT, encoding histidine kinase (HPt) is also involved. Active two component system of ysa-ysp T3SS regulates ysaE-a member of AraC XylS family and sycB a transcriptional regulator with chaperone activity (89, 90). A recent study showed YsrS when mutated or its orthologue like rcsB, the genes within the Ysa PI gets inactivated [91]. Such cocclusion was based on monitoring of lacZYA expression in a construct where the promoter of rcsB was mutated [91].

**Ysc-yop system:**

The Ysc-Yop system is the more extensively studied Type threesecretion system in *Y. enterocolitica*. The external stimuli like Ca2+ and alkaline pH activates the two component system CpX-A and CpX-R. The phosphorylated CPX- R in a similar way to YsrS YsR and YsrT except for the fact the histidine phosphotransferase domain is intact within the CPX- R. The activated CPX- R-P (phosphorylated) transcribes the genes in pYV plasmid. This triggers transcription of proteins associated with T3SS in the pYV system. The pYV system produces the translocators (YopD, YopB) and effectors guided (YopN, YopE, YopT etc) by respective chaperones (LcrH or Syc D, SycE, Syc N) which are regulated by LcrG and LcrV acting as tip proteins. Additionaly the apparatus protein like YscJ, YscD, YscC, YscU and YscF are involved in construction of the needlecomplex on contact with host. The translocation of the
effectors and translocators take place via hollow conduit and is energized by the ATPase YscN. YscL one of the regulator proteins tethers and controls the functionality of YscN ATPase. The presence of separate machinery for export of these effectors and translocators clearly suggest the organism uses two different independent type three secretion systems for delivery of toxins into host cell. The Ysa- ysp system is associated with systemic phase and the ysc yop system is associated with gastro intestinal phase of the infection caused by this microorganism. YopN and YopE are common effectors that are transloacted via both the type three secretion systems. In Yersinia enterocolitica, these two different Type three secretion systems account for the complete virulence of the pathogen. A detailed description is given in Figure 8.

The pantothenate pathway in Yersinia enterocolitica is responsible for production of CoA and acetyl CoA. The phosphopanteheine adenyl transferase is one of the major catalytic enzymes in the process that catalyses the penultimate rate limiting step of CoA biosynthesis. Although evidences are rare but there are reports of correlation between the pantothenate pathway and T3SS. Pantothenate moieties are used by Acyl carrier proteins (ACP). The Ysa-Ysp locus consists of an ACP viz acpY. The presence of such gene within the locus might indicate the direct corelation between such metabolic (pantothenate CoA) and virulence (T3SS) pathway. Such proposed hypothesis of crosstalk needs careful and detailed experimentation. Regulation is also mediated by acetyl CoA at the cellular level in response to intercellular variation of CoA and ACoA via TCA and glyoxalate pathway. Genes like aceF aceG are likely to play important part in regulation via YsaE thereby controlling transcription, although such mechanism is not deciphered fully (Figure 8).
Fig 3.2 Genomic organisation of ysa-ysp TTSS in Yersinia enterocolitica
Fig 3.3 Hypothetical model showing correlation between Ysa-Ysp, Ysc-Yop T3SS and Pantothenate CoA biosynthesis pathways in Y. enterocolitica ATCC 51871, similar to Y. enterocolitica 0:8 8081.
**Pseudomonas aeruginosa:**

Type three secretion system is regulated in *P. aeruginosa* is mainly through regulatory proteins at secretion initiation of secretion and transcriptional level [92, 93]. Like other orthologues the main activation of T3SS is controlled by ExsA- a member of AraC/Xyls family. Although the exact mechanism is not well known, ExsA ExsC ExsD and ExsE are major proteins that regulate the activation of secretion at the transcriptional level. They use a mechanism called ‘catch and release’. under secretory inhibition condition, the ExsA binds with ExsD, a inhibitor which represses expression of ExsA and other activator proteins that initiates transcription of T3SS genes. ExsC an anti anti transcriptional (activator) disrupts the ExsA-ExsD complex formation and allows the binding of ExsA to its promoter, thereby allowing transcription of T3SS genes. Activation of T3SS leads to expression of different effectors like ExoS, ExoT, ExoY & ExoU (Pa-14only) at basal level until contact with host cell. They are channelized through the injectisome via specific chaperones SpcS and SpcU (Pa-14only). The regulator proteins PcrG controls PcrV which is a hydrophilic translocator. The PopB and PopD acts as hydrophobic translocators that are transported via chaperone PcrH.

![Fig 3.4 T3SS in Pseudomonas aeruginosa: left transcriptional control and right the overview](image)
Figure 3.5 Correlation network between Pantothenate pathway and Type three secretion pathways in Pseudomonas aeruginosa
At the global scale, the two proteins CyaA and CyaB regulate expression from T3SS promoters via Vfr, a global regulatory protein with homology to the *Escherichia coli* cAMP receptor protein (CRP). In the Gac system, the two-component sensors RetS and LadS transduce environmental signals to modulate the two-component GacA/GacS system, which in turn regulates expression of T3SS genes. RetS primes *P. aeruginosa* for acute infections by inducing T3SS genes and normal piliation and represses genes that promote biofilm growth. LadS acts in a reciprocal manner; it down regulates type III secretion system [94-98]. It is thought that the metabolic signal derived from acetyl-CoA controls the *P. aeruginosa* T3SS via CyaB [99]. Apart from acetyl-CoA, tryptophan [99] and histidine [100], also regulates the T3SS in *Pseudomonas*. AcCoA is synthesized from CoA. CoaD or phosphopantethiene adenylyltransferase plays a pivotal role in synthesis of CoA along with dephosphocoenzymeA kinase. Phosphopantethiene adenylyltransferase (PPAT) is an enzyme that catalyzes the reversible reaction of converting ATP and pantethiene to dephosphocoenzyme and pyrophosphate. The enzyme has overlapping site in substrate binding and inhibitory states indicating allostERICITY. Previous study in *E. coli* indicated that acetyl-CoA binds with PPAT. In *P. aeruginosa*, the structure of PPAT with acetyl-CoA is solved and characterised biophysically. Additionally, it is established experimentally the catalytic activity is controlled by feedback regulation by CoA and Acetyl CoA [185]. This indicates direct co-relation about the regulation of pantothenate and type three secretion system. In vitro studies provided insight into the physiology of the less understood *P. aeruginosa* infection via T3SS. Ca2+ along with some important amino acids is important in triggering T3SS as verified experimentally [101, 102 and 103]. A detailed summary of this is depicted in Figure 11.