Controlling virulence in *Yersinia pseudotuberculosis* through accumulation of phosphorylated CpxR

Edvin Jose Thanikkal
To my family....
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Abstract

Like many Gram-negative bacteria, the food-borne pathogen *Yersinia pseudotuberculosis* harbours different regulatory mechanisms to maintain an intact bacterial envelope especially during exposure to extracytoplasmic stress (ECS). The CpxA-CpxR two component regulatory system is one such ECS-responsive regulatory mechanism. Activation of CpxA-CpxR two-component regulatory system (TCRS) accumulates phosphorylated CpxR (CpxR~P), which not only up-regulates various factors that are designed to maintain envelope integrity, but also down-regulates key determinants of bacterial virulence.

*Y. pseudotuberculosis* establishes close host cell contact in part through the expression of the invasin adhesin. Invasin expression is positively regulated by the transcriptional regulator RovA, which in turn is negatively regulated in response to nutrient stress by a second transcriptional regulator RovM. In *Y. pseudotuberculosis*, loss of CpxA phosphatase activity accumulates CpxR~P, and this represses both *rovA* and *inv* transcription directly, or indirectly via activation of *rovM* transcription. It is now of interest to understand the molecular mechanism behind how CpxR~P regulates gene transcription both positively and negatively.

A type III secretion system (T3SS) is a highly conserved multi-protein secretion system used by many Gram-negative bacteria to secrete protein cargo that counteracts the effects of a host cell emitted anti-bacterial activity. A typical set of proteins that make-up a functional T3SS includes structural proteins, translocators, effectors and regulatory proteins. Accumulation of CpxR~P was shown to repress the plasmid encoded Ysc-Yop T3SS of *Y. pseudotuberculosis*. Although yet to be confirmed experimentally, promoter-CpxR~P binding studies indicate multiple modes of regulatory control that for example, could influence levels of the plasmid-encoded Ysc-Yop system transcriptional activator, LcrF, and the chromosomal encoded negative regulators YmoA and YtxR.

Regulatory processes of TCRS involve transient molecular interactions between different proteins and also protein with DNA. Protein-protein interaction studies using the BACTH assay showed that it can be useful in analysing the molecular interactions involving the N-terminal domain of CpxR, while the λcl homodimerization assay can be useful in analysing molecular interactions involving the C-terminal domain of CpxR. Therefore, in combination with other biochemical and physiological tests, these hybrid-based assays can be useful in dissecting molecular contacts that can be helpful in exploring the mechanism behind CpxR~P mediated transcriptional regulation.

In conclusion, this work uncovered direct involvement of CpxR~P in down-regulating virulence in *Yersinia pseudotuberculosis*. It also utilised genetic mutation and explored different protein-protein interaction assays to begin to investigate the mechanism behind the positive and negative regulation of gene expression mediated through active CpxR~P.
Abbreviations

T3SS  Type III secretion system
MDRS  Multiple drug resistant strain
M cell  Microfold cell
MLN  Mesenteric lymph nodes
PPs  Payers patches
YadA  Yersinia adhesin A
Ail  Attachment-invasion locus
LPS  Lipopolysaccharide
OM  Outer membrane
Yops  Yersinia outer proteins
Syc  Specific Yop chaperone
ECM  Extracellular matrix
Ysc  Yersinia secretion
IM  Inner membrane
LCR  Low calcium response
RovA  Regulator of virulence A
MarR  Multiple antibiotic resistance regulator
H-NS  Histone-like nucleoid-structuring
YmoA  Yersinia modulator A
Hha  High hemolysin activity
Csr  Carbon storage regulator
cAMP  Cyclic adenosine monophosphate
Crp  cAMP receptor protein
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<th>Abbreviation</th>
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<tr>
<td>RR</td>
<td>Response regulator</td>
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<tr>
<td>CCR</td>
<td>Carbon catabolite repression</td>
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<td>TCRS</td>
<td>Two component regulatory system</td>
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<td>ECS</td>
<td>Extracytoplasmic stress</td>
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<td>Cpx</td>
<td>Conjugative plasmid expression</td>
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<td>Regulation of capsular polysaccharide synthesis</td>
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<td>Psp</td>
<td>Phage shock protein</td>
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<tr>
<td>DHp</td>
<td>Dimerization histidine phosphotransfer</td>
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<td>CA</td>
<td>C-terminal catalytic ATP-binding</td>
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<td>OMP</td>
<td>Outer membrane protein</td>
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<td>CpxR~P</td>
<td>Phosphorylated CpxR</td>
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<td>CPS</td>
<td>Capsular polysaccharide synthesis</td>
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<td>Ysa</td>
<td>Yersinia secretion apparatus</td>
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<td>Ysp</td>
<td>Yersinia secreted proteins</td>
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Papers included in the thesis:

This thesis is based on the following publications and manuscripts referred to by their roman numerical (I – IV).


II. Thanikkal EJ, Obi IR, Liu J, Gurung JM, Dersch P and Francis MS, The *Yersinia pseudotuberculosis* Cpx envelope stress system contributes to transcription activation of *rovM* (Manuscript)


IV. Thanikkal EJ, Mangu JC, Francis MS, Interactions of the CpxA sensor kinase and cognate CpxR response regulator from *Yersinia pseudotuberculosis*. BMC Research Notes 2012; 5:536.

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1. Introduction

1.1 The genus *Yersinia*

*Yersinia* is a Gram negative, facultative bacteria that belongs to the Enterobacteriaceae family. Among the seventeen known *Yersinia* species, three have been shown to be virulent to humans – *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis* [1]. *Y. pestis* was first isolated by Swiss-French bacteriologist Alexandre Yersin (1863-1943) and the genus was named after his discovery [2]. All *Yersinia* species can grow in nutrient rich agar and the optimum growth temperature is between 25°C to 29°C, but it is possible for them to survive between a temperature range of 4°C to 42°C [3]. The enteropathogenic species *Y. enterocolitica* and *Y. pseudotuberculosis* enters the human body mainly through contaminated food and water causing a variety of gut and lymph associated diseases [4, 5]. The means by which *Y. pestis* enters humans is either via the bite of an infected flea or by aerosol inhalation and this often lead to the emergence of septicemic, pneumonic or bubonic plague [3, 6]. Bubonic plague infection is considered to be one of the deadliest infectious diseases known to society [7].

The three human virulent strains *Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis* carry a common 70-kb plasmid that is essential for sustained bacterial replication in host tissues [8]. This plasmid encodes a type III secretion system (T3SS) and numerous secretion substrates that are involved in inhibiting phagocytosis and proinflammatory cytokine production [6, 9].

1.2 History of plague

Plague is caused by *Y. pestis* and has historically been divided into three pandemics. The first pandemic is called as the Justinian plague which occurred between 6th and 8th century. This plague began from the African continent and is thought to have lasted for 250 years [10]. The second pandemic is referred to as Medieval plague or Black Death which happened...
between 14\textsuperscript{th} and 19\textsuperscript{th} century. It began in China and entered Europe as a consequence of China being one of the busiest trading nations in the world. This pandemic killed nearly 30\% of the population in Europe. The third pandemic also started in China in the second half of 19\textsuperscript{th} century and is still continuing [10, 11]. These three pandemics were associated with three different biovars of \textit{Y. pestis} called Antiqua, Medievalis and Orientalis respectively [3]. Plague is still an endemic in many parts of the world, and Africa accounts for more than 90 percent of human plague cases reported worldwide [12]. The recent emergence of multiple drug resistant strains (MDRS) in Madagascar shows the importance of achieving a better understanding of the disease [13, 14].

1.3 Pathogenesis of \textit{Yersinia pestis}

Plague is a zoonotic disease that can be contracted from infected animals or their parasites. Most often the disease is acquired by a flea bite. Many flea species are likely to be plague vectors and the most common vector is \textit{Xenopsylla cheopis}, associated with oriental rat. The fleas are infected on taking a blood meal containing \textit{Y. pestis}. Subsequent multiplication of \textit{Y. pestis} blocks the valve connecting the oesophagus and midgut of the flea. At the same time the product of the \textit{pla} gene encoded on pPCP1 plasmid, one of the other two unique plasmids of \textit{Y. pestis}, prevents ingestion of blood by the flea. The blocked fleas eat more aggressively and regurgitate the bacteria while making persistent efforts to feed [3, 12, 15].

The three primary clinical forms of \textit{Y. pestis} infections are bubonic plague, primary septicemic plague and primary pneumonic plague. Bubonic plague is characterized by swelling or regional inflammation in one or several lymph nodes. It is the most common form of plague and has a mortality rate between 30\% to 75\% if left untreated. Primary septicemic plague is caused by an infection in the bloodstream in the apparent absence of a primary lymphadenopathy. Pneumonic plague is caused by the inhalation of infectious droplets. In most cases, primary pneumonic plague is usually fatal without treatment [11, 16, 17].
1.4 Evolution of Yersinia pestis from Yersinia pseudotuberculosis

Accumulated molecular and genomic evidence showed that *Y. pseudotuberculosis* is the ancestor of *Y. pestis*, with *Y. pestis* suggested to have evolved some 1500 to 20000 years ago [18] [19]. Despite the fact that *Y. pestis* is evolved from *Y. pseudotuberculosis*, exceptional differences exist between them in terms of disease pattern and epidemiology. *Y. pseudotuberculosis* transmission occurs through contaminated food and water, while *Y. pestis* largely relies on blood feeding fleas for transmission. In terms of epidemiology, *Y. pseudotuberculosis* most often causes mild self limiting local enteric diseases in mammals, while *Y. pestis* is highly invasive and virulent [19, 20]. Genetically, *Y. pestis* carries two additional plasmids termed pPCP1 and pMT1 that are significant in terms of capsule formation, tissue invasion and infection of the flea vector [19]. Hence, these two plasmids contain genes that specifically contributed to evolution of flea-borne transmission route. For example the phospholipase D activity encoded by *ymt* gene encoded by the plasmid pMT1 is required for the survival of *Y. pestis* in the midgut of flea [21]. Furthermore, the plasminogen activator/protease that is encoded by *pla* gene on the pPCP1 plasmid is important for bacterial dissemination from the actual flea bite site [22]. It is necessary to point out that gene loss also played a significant role in the evolution of *Y. pestis*. The presence of an abundance of pseudogenes and movable transposable genetic elements was also observed in *Y. pestis* relative to *Y. pseudotuberculosis* [20, 23, 24].

1.5 Infection route of Yersinia pseudotuberculosis

*Y. pseudotuberculosis* infections are typically initiated by consuming contaminated food and water. Various animals such as pigs and buffalo are considered as primary environmental reservoirs of *Y. pseudotuberculosis* [25]. Upon infection, *Y. pseudotuberculosis* can survive the acidic conditions in the stomach and reach the small intestine [26, 27]. *Y. pseudotuberculosis*
invades the intestinal barrier by passing through the M cells (Microfold cells) present in the small intestine. M cells are specialized antigen sampling cells found in follicle associated epithelium [28]. On reaching the intestinal barrier, bacteria start replicating in the lymphoid follicles which are called Payer patches (PPs). Mesenteric lymphadenitis can occur if Y. pseudotuberculosis spread to mesenteric lymph nodes (MLN). In rodents, Y. pseudotuberculosis from the intestinal barrier can disseminate to major lymphatic organs like lungs, liver and spleen. In the blood stream, Y. pseudotuberculosis can cause septicemia even though this is rare in humans [16].
Figure 1: Infection route of *Yersinia pseudotuberculosis*. Bacteria enter the human body through contaminated food and water. There they can reach PPs by passing through the M-cells. Bacteria can proliferate in PPs and disseminate to mesenteric lymph node and further to lungs, liver and spleen.
1.6 Yersinia pseudotuberculosis as a model organism

Y. pestis, the causative of infamous plague was evolved from Y. pseudotuberculosis. The genomic similarity between Y. pestis and Y. pseudotuberculosis is very high (97% nucleotide sequence identity) and their rRNA sequences are identical [29, 30]. They share many common important properties like tropism for lymphoid tissues, the presence of 70 kb virulence plasmid (known as pCD1 in Y. pestis, pIB1 in Y. pseudotuberculosis and pYV in Y. enterocolitica) that is being used for delivering effector proteins into the host cytoplasm, an ability to kill macrophages, resistance to compliment mediated killing and most significantly they can efficiently infect mice and cause much of the pathology seen by plague infections in humans [8, 31, 32]. Furthermore the genomes of several Y. pseudotuberculosis isolates are fully sequenced. They are easy to handle, culture and make clear genetic manipulations. Thus, taken all together this makes Y. pseudotuberculosis an ideal model for studying Y. pestis pathogenesis.

1.7 Important virulence factors of Yersinia pseudotuberculosis in the early phase of infection

Y. pseudotuberculosis enters the human body through contaminated food and water. In order to survive and invade successfully in the host, bacteria need to produce virulence factors that enable them to proliferate and defend themselves from the host immune response. The virulence factors of enteropathogenic Yersinia are either encoded on the 70 kb virulence plasmid or on the chromosome [32-35]. Notable virulence factors include various adhesins and internalization factors like invasin, Yersinia adhesin A (YadA), and attachment-invasion locus (Ail) along with survival and immune evasion factors such as the Yop effector proteins injected by T3SS that are encoded in the 70 kb virulence plasmid pIB1 as shown in figure 2 [1, 8, 36].
Invasin is an adhesin protein present in the outer membrane (OM) of the enteropathogenic *Yersinia* species - *Y. pseudotuberculosis* and *Y. enterocolitica*. In *Y. pseudotuberculosis*, the 103 kDa invasin is chromosomally encoded by the *inv* gene [38-40]. In both enteropathogenic *yersinia* species maximal expression of invasin was observed on growing the bacteria in rich medium until late stationary phase at moderate temperature (20°C-28°C) [41-44].

Invasin is considered to be the first adhesin that becomes activated during the early phase of infection. Invasin is responsible for the internalization of *Y. pseudotuberculosis* into the host epithelial cells and especially M cells - present in the small intestine, and this is mediated by binding to multiple β1 integrins present on the host epithelial cell surface [1, 45]. Binding of invasin to integrins induces their clustering, which in turn
remodels the actin cytoskeleton leading to the internalization of *Y. pseudotuberculosis* into non-phagocytic cells by a process known as the zipper invasion mechanism [46]. Adhesion and uptake of *Y. pseudotuberculosis* requires a high density of invasin present in the bacterial OM and a high density of integrins present in the epithelial cell membrane. If both invasin and integrin levels are low, just adherence of *Y. pseudotuberculosis* occurs, not internalization [47, 48].

Intragastric infection of mice with *Y. pseudotuberculosis* lacking *inv* gene reduced the amount of bacteria at PPs in the early phase of infection, but this did not reduce the virulence of *Y. pseudotuberculosis* mutant strain lacking *inv* on comparison with parent strain after several days of post infection, showing the importance of invasin in the early phase of infection [43, 49, 50]. Due to the presence of an IS200 element within the *inv* gene, *Y. pestis* cannot express invasin and this might be one of the reasons behind the alternative route of infection in *Y. pestis* [51, 52].

### 1.7.2 Yersinia adhesin A (YadA)

YadA is a trimeric adhesin protein present in the OM of enteropathogenic *Yersinia* species *Y. pseudotuberculosis* and *Y. enterocolitica*. The gene *yadA* is encoded in the 70 kb virulence plasmid pIB1 [53, 54]. Depending on the *Yersinia* species, the molecular mass of YadA protein varies between 160 to 250 kDa [55]. Adherence of enteric *Yersinia* strains to professional phagocytes, epithelial cells and proteins in the extracellular matrix (ECM) like collagen and fibronectin are mediated by YadA [53, 55]. Agglutination and hemagglutination are mediated by YadA in *Y. pseudotuberculosis* enabling the bacteria to sustain neutrophil attacks and microcolony formation in the lymphatic tissue [55-57]. YadA protein produced by *Y. pseudotuberculosis* preferentially binds to fibronectin due to the presence of an additional 31 amino acids absent from YadA produced by *Y. enterocolitica*. In contrast, YadA present in the *Y. pseudotuberculosis* does not bind to collagen and laminin [56]. In *Y. pseudotuberculosis*, entry
to epithelial cells in the absence of invasin activity is facilitated by the YadA adhesin. YadA binds to β integrins indirectly by means of ECM molecules present on epithelial cells [55]. Note that the yadA gene present in Y. pestis is a pseudogene due to a frame shift mutation caused by deletion of a single nucleotide and this is due to the switching of Y. pestis from an enteric lifestyle to mammalian blood borne lifestyle [52, 58].

1.7.3 Attachment-invasion locus (Ail)

Ail is a 17 kDa adhesin present in the OM of all the three human pathogenic Yersinia species and in all cases is encoded in the chromosome [59, 60]. Ail is involved in serum resistance by all three bacterial species [61]. Due to the small size of Ail, it is masked by the O-antigen present on the lipopolysaccharide (LPS) in Y. pseudotuberculosis and Y. enterocolitica. Ail seems to play an active role when the bacteria is having rough LPS [62]. The LPS that lacks O-antigen is called as rough LPS [63, 64].

1.7.4 Type 3 secretion system (T3SS)

After crossing the intestinal barrier through M cells, enteropathogenic Yersinia encounters a host immune response in the PPs. Pathogenic Yersinia species make use of a T3SS in order to overcome the host immune response and establish a bacterial infection [65]. T3SS is a syringe like multi-protein complex spanning the bacterial membrane for delivering cytotoxic effectors from the bacterial cytoplasm to the host cytosol [34]. At least 25 proteins are required to make this T3SS apparatus and these are generally called Ysc (Yersinia secretion) proteins [66]. The proteins secreted by T3SS are called Yersinia outer proteins (Yops). Yops are divided into translocators (YopB, YopD and LcrV), effectors (YopE, YopH, YpkA/YopO, YopJ/P, YopT and YopM) and regulators (YopK/YopQ and YopE) [67-69]. The common virulence plasmid in Yersinia encodes genes for (i) T3SS apparatus, (ii) translocators, (iii) effectors, (iv) regulators of secretion and translocation
along with (v) Syc (specific yop chaperone) proteins that functions as chaperones for effectors and translocators [8, 70-72]. The schematic representation of T3SS activity during bacteria-host cell contact is shown in figure 4.

1.7.4.1 T3SS apparatus

The ‘syringe like’ T3SS apparatus consists of basal body embedded in the bacterial membrane that assemble together to span the inner membrane (IM), periplasm and OM as shown in figure 3. The basal structure of the T3SS apparatus is built up from different Ysc proteins [36, 73, 74]. It is suggested that the T3SS apparatus - which is also termed the injectosome - has an evolutionary origin from the bacterial flagella system based on genetic homology studies [75].

![Figure 3: Schematic representation of T3SS apparatus.](image)

1.7.4.2 Yop translocators, effectors and regulators

The hydrophobic YopB and YopD translocators are assembled at the interface between the hydrophilic needle tip complex protein LcrV and the host cell plasma membrane (figure 4) [72]. Thus a complex of YopB and YopD together with LcrV are involved in pore formation within the
eukaryotic host cell membrane [67]. This pore formation opens up a gateway for the effector proteins YopE, YopH, YpkA/YopO, YopT, YopJ/P and YopM to be directly injected into the host cytoplasm [68, 69]. When located inside the host cell, phagocytosis by macrophages and neutrophils are counteracted by the activities of effector proteins YopE, YopH, YopO and YopT [76, 77]. Additionally YopJ/P inhibits cytokine production and induces macrophage apoptosis [78-80], where as YopM inhibits Caspase-1 activity and prevents the inflammatory cell death program pyroptosis of the macrophage. [81-83]. Finally YopK has a regulatory role for Yop translocation and secretion. YopK is known to interact with YopD and this may regulate the ratio of YopB and YopD in the assembled pore [84-86]. Similar to YopK, YopE has also been reported to be involved in regulation of Yop translocation in addition to its role in anti-phagocytosis [36, 85, 87].

1.7.4.3 Regulation of T3SS in Yersinia

Many bacteria use temperature as an important regulator for virulence due to elevated temperature inside the mammalian host [8, 88]. The in vitro activity of T3SS is primarily controlled by temperature and calcium concentration [89-91]. Bacteria grown in the presence of calcium at 37°C, induces only very low expression levels of T3SS genes to assemble some T3SS apparati [89]. On the other hand depletion of calcium from the growth medium ceases bacterial growth and triggers up regulation of T3SS genes with the subsequent secretion of Yops [89, 90, 92, 93]. This growth restricting phenomenon is termed the low calcium response (LCR) [94]. Activation of T3SS is mediated by an increased expression of the positive transcriptional regulator low calcium response F (LcrF). LcrF is an AraC-like transcriptional activator [88, 95]. Yersinia requires target cell contact for polarized translocation of Yop effectors to the cytoplasm of the host in vivo [92, 96, 97]. Several negative regulators are involved in preventing T3SS. LcrQ is a known anti-activator important for negative regulation of T3SS. The type III secretion of LcrQ is a signal for bacteria to secrete Yops at 37°C under low calcium condition [98-100].
Figure 4: T3SS activity during bacteria-host cell contact. Enteropathogenic Yersinia encounters a host immune response on crossing the intestinal epithelial barrier. Yops are secreted into the host cells upon tight cell contact, initially mediated by Invasin and YadA. This image is modified from [9].

1.8 Important virulence regulators in the early phase of infection

1.8.1 RovA the transcriptional regulator of inv

The regulator of virulence A (RovA) is a transcriptional regulator that positively regulates gene expression of the invasin adhesin (figure 5) in response to environmental signals [41]. RovA belongs to the MarR (multiple antibiotic resistance regulator)-type family of winged helix transcription factors [101]. It was first identified in Y. enterocolitica as a regulator of inv -
encoding for invasin using a transposon mutagenesis screen [102]. Shortly afterwards, it was identified in *Y. pseudotuberculosis* [41]. In all pathogenic *Yersinia* species, RovA is considered as a global transcriptional regulator due to its role in coordinating multiple metabolic, virulence and stress genes in response to signals from the environment inside the host [103].

A concentration dependent mechanism governs RovA expression levels. An auto-regulatory feedback loop controls the expression level of RovA inside the bacteria. The transcription of *rovA* occurs from two different promoters P1 and P2 located 76 and 343 nucleotides upstream of the translational start site. Autoactivation of *rovA* transcription is mediated by binding of RovA to a high affinity binding site located in an upstream region of P2 promoter. However, high intracellular levels of RovA blocks autoactivation by binding to the low affinity site located downstream of the P1 promoter and thereby interfering with the function of RNA polymerase [44, 104]. Expression of *rovA* is strictly controlled by different parameters like temperature, availability of nutrients and bacterial growth phase. Maximal RovA synthesis was observed at moderate temperature in complex media at stationary phase [41, 44]. RovA activity is also controlled post-transcriptionally by acting as an intrinsic thermometer that senses a temperature shift by reversible alterations in protein conformation and thereby controlling its DNA-binding capacity [105].

Even though oral infection of mice with *Y. pseudotuberculosis* strains lacking *rovA* are able to establish an infection in PPs, only few bacteria were recovered from PPs in comparison with the wild type strain [104]. Moreover, mutant *Y. pseudotuberculosis* strain lacking *rovA* was severely attenuated in its ability to reach liver and spleen [104]. In *Y. pestis*, RovA is also required for virulence although they lack a functional *inv* gene, showing that RovA can have alternative roles in virulence. Intranasal and intraperitoneal inoculation of mice with *Y. pestis* lacking *rovA* has shown only a slight difference in virulence, but at the same time subcutaneous inoculation shows
a drastic decrease in virulence indicating the significance of RovA in bubonic plague more than pneumonic plague [106].

Additionally, Expression of \textit{rovA} was found to be regulated by other factors that include nucleoid –associated proteins like H-NS and YmoA, RovM, carbon storage regulatory system (CsrABC), cAMP receptor protein (Crp) and two response regulators (RRs) UvrY and PhoP [43, 104, 107-109].

1.8.2 The nucleoid-associated protein H-NS

The histone-like nucleoid-structuring protein (H-NS) functions as an architectural protein and a global modulator of gene expression in enteric bacteria [110]. H-NS is known to silence transcription by binding to AT-rich regions and the precise mechanism is not known [111-115]. In \textit{Y. pseudotuberculosis}, expression of \textit{rovA} and \textit{inv} are repressed by H-NS by binding to the promoters of \textit{rovA} and \textit{inv} at 37°C [44]. Binding region of H-NS in the regulatory region of the \textit{rovA} and \textit{inv} promoters overlaps with the binding region of RovA. Hence, H-NS mediated silencing of \textit{inv} and \textit{rovA} is antagonised by RovA because of its strong affinity towards the target promoter sequences of \textit{inv} and \textit{rovA} at 25°C [44].

1.8.3 The nucleoid-associated/ histone like protein-YmoA

The \textit{Yersinia} modulator A (YmoA) protein is a low molecular mass protein (8 kDa) that belongs to the family of Hha/YmoA proteins [95, 116]. A homolog of YmoA is high hemolysin activity (Hha) protein and was identified in \textit{Salmonella} and \textit{Escherichia coli} (\textit{E. coli}) [117]. The proteins belonging to Hha/YmoA family have been shown to interact with H-NS or its homologues like StpA and regulate the expression of virulent genes [118-120].

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The repression of inv mediated through YmoA was observed in *Y. enterocolitica* [121]. Protein-protein interaction studies in *Y. enterocolitica* showed interaction between YmoA and H-NS protein [118], predicting a possible role for YmoA in H-NS mediated repression of *rovA* [103, 122]. On the contrary, removal of YmoA in *Y. pseudotuberculosis* enhanced RovM expression levels leading to subsequent decrease in *rovA* and *inv* [43], thus suggesting different possible roles for YmoA in enteropathogenic *Yersinia*. Meanwhile, the stability of YmoA was also varied among enteropathogenic *Yersinia* at different temperatures. The stability of YmoA at lower temperature (15°C) as well as 37°C remained good in *Y. enterocolitica* [123] but at the same time, YmoA gets degraded at 37°C in both *Y. pestis* [124] and *Y. pseudotuberculosis* [43]. Thus, a possible temperature dependent role for YmoA in controlling *rovA* expression is suggested in *Y. pseudotuberculosis*.

### 1.8.4 RovM the transcriptional regulator of *rovA*

RovM belongs to the LysR family of transcriptional regulators. RovM is homologous to virulence regulators like HexA in *Photorhabdus temperata* and PecT in *Erwinia chrysanthemi* and *Erwinia carotovora*. Enhanced expression of RovM was observed in *Y. pseudotuberculosis* strains growing in minimal media and this is mediated through a complex PhoP-Crp-CsrABC-RovM regulatory cascade [104, 107-109]. RovM inhibits transcription of *rovA* in *Y. pseudotuberculosis* (figure 5). In vitro protein-DNA binding studies showed that RovM can directly bind near the P1 promoter of *rovA* and repress its expression [104]. RovM requires the presence of H-NS to repress *rovA* transcription and, at the same time is also needed by H-NS for silencing *rovA* [104].

Deletion of *rovM* leads to an increased expression of *rovA* that inturn induced expression levels of the invasin adhesin leading to an increase in *Y. pseudotuberculosis* uptake upon infecting cultured human epithelial cells [104]. Oral infection of mice with a *Y. pseudotuberculosis* strain lacking *rovM* revealed more bacteria in PPs, mesenteric lymph nodes, liver and
spleen on comparison with the wild type strain. On the contrary, strains over expressing RovM were attenuated for virulence in mice and a significantly lower number of bacteria were found in lymphatic tissue and no bacteria were detected in organs of mice [104].

1.8.4.1 Csr system

Carbon storage regulatory (Csr) system is an important post-transcriptional regulatory system that controls stability and translation of mRNA that is involved in virulence, stress adaptation and metabolic function in Yersinia [125, 126]. It consists of the RNA binding protein CsrA and two non-coding small regulatory RNAs CsrB and CsrC [107]. CsrA binds on shine-dalgarno sequence to inhibit the initiation of translation and in most cases facilitates mRNA decay [127]. The small regulatory RNAs CsrB and CsrC are capable of binding to and sequestering CsrA [107]. Complex secondary structures are formed by the small regulatory RNAs CsrB and CsrC with multiple 5’-GGA/RGGA-3’ motifs located in the loop region of the predicted RNA hairpin and these sequestering limits CsrA available to bind to mRNA targets [127-129].

In Y. pseudotuberculosis, removal of the csrA gene or overexpression of the Csr-RNAs CsrB and CsrC products lead to an increased expression of RovA and invasin at the same time as RovM expression levels were low. Hence, expression of RovA influenced by CsrA is indirectly mediated through RovM [107] (figure 5). Interestingly, differential expression of CsrC was observed by growing Y. pseudotuberculosis in complex media and minimal media and this lead to a possible link between the Crp protein and virulence control in Yersinia [108].

1.8.4.2 Crp

The cAMP receptor protein (Crp) in the Enterobacteriaceae family acts as a global regulatory protein that is involved in gene transcription
control based on glucose supply [130, 131]. Crp forms a complex with cyclic adenosine monophosphate (cAMP) and this complex can control gene expression by binding in the promoter region of different target genes at a universally conserved binding box for Crp-cAMP [132, 133]. The presence of glucose in the medium decreases cAMP production as well as Crp levels by a process known as carbon catabolite repression (CCR) that prevents bacteria from activating gene expression specifically involved in utilization of alternative sugars [134, 135]. Glucose present in the medium inhibits adenyl cyclase enzyme activity which is responsible for converting Adenosine triphosphate (ATP) to cAMP [134, 136].

A substantial decrease in virulence (>15000 fold) was observed in mice infected subcutaneously with *Y. pestis* harbouring a disrupted *crp* gene [137]. *Y. enterocolitica* lacking *crp* showed a decrease in virulence during oral infection in mice. In *Y. enterocolitica* the genes encoding components of the Ysc-Yop T3SS are affected by cAMP-Crp complex and this is probably one reason for the virulence attenuation [138]. Oral infection in mice with *Y. pseudotuberculosis* lacking *crp* gene revealed that bacteria were unable to gain access to deeper tissues like liver and spleen. Phenotypic analysis in *Y. pseudotuberculosis* shows that loss of *crp* affects expression levels of the regulatory RNAs CsrB and CsrC leading to increased levels of RovM and thereby repression of RovA and invasin [108]. Thus, Crp plays a significant role in *Y. pseudotuberculosis* by correlating nutrient status with virulence that is important for optimizing bacterial fitness and infection efficiency during their infectious life cycle. Evidently, the RR UvrY (discussed in section 1.8.4.3) is involved in Crp-mediated control of the virulence gene cascade CsrABC-RovM-RovA-Invasin [107, 108].

### 1.8.4.3 BarA-UvrY

Two component regulatory systems (TCRS) are involved in transducing external signals into the cell and enable a reprogramming of gene expression. They are widely present in bacteria and respond to diverse
environmental signals and stimulations [139, 140]. BarA-UvrY is one such TCRS present in several Gram negative bacteria [140]. BarA functions as a conserved sensor kinase and UvrY functions as its cognate RR [141-144].

In *Y. pseudotuberculosis*, Crp represses transcription of UvrY and thereby indirectly decreases levels of CsrB RNA, since UvrY is known to activate transcription of the *csrB* gene [108]. During intranasal infection of *Y. pestis* in mice, expression of UvrY was only detected in *Y. pestis* located in the lung but not in liver and spleen [145]. The signals that activate BarA-UvrY TCRS in *Y. pseudotuberculosis* is unknown. Based on UvrY activation in lungs during *Y. pestis* infection, it is possible that BarA-UvrY system might be sensing metabolites that are present only in certain host niches [108].

### 1.8.4.4 PhoP-PhoQ

The PhoP-PhoQ is a TCRS that plays an important role in bacterial virulence. PhoQ functions as a membrane bound sensor kinase and PhoP is the corresponding RR present in the cytoplasm. The PhoQ sensor kinase activates and donates a phosphate group to activate the PhoP RR in accordance with signals like acidic pH, antimicrobial peptides secreted by the host and low levels of environmental magnesium, calcium and manganese. [109, 146].

Recent studies show that PhoP activates expression of *csrC* RNA in *Y. pseudotuberculosis* by direct binding to DNA within the promoter region. An increase in expression of *csrC* RNA enhances RovA expression through the CsrABC-RovM signaling cascade as shown in figure 5 since it titrates away CsrA [109]. Accordingly, *Y. pseudotuberculosis* strain lacking *phoP* gene is attenuated in a mice infected orally [147].
1.9 Extracytoplasmic stress responses

Extracytoplasmic stress (ECS) can be explained as external physical and/or chemical environments that compromise bacterial envelope integrity and/or protein folding in the periplasm. The bacterial ECS response is an important adaptive mechanism that has the goal to alleviate defects in the bacterial membrane integrity and periplasmic protein folding during exposure to one or more ECSs [148, 149]. A few notable ECS response pathways are briefly discussed in this thesis; these are the conjugative plasmid expression (CpxAR) TCRS, the alternative sigma factor $\sigma^E$, the regulation of capsular polysaccharide synthesis (Rcs) phosphorelay system
and the phage shock protein (PSP) system. Crucially, recent studies have revealed that ECS responsiveness is not only important in maintaining bacterial membrane integrity, but is also involved in the regulation of virulence expression [149-151].

1.9.1 CpxAR two-component regulatory system

The Cpx pathway was first identified and characterized by McEwen and Silverman during a mutagenesis screen for chromosomally encoded genes that affect the function of conjugative F plasmid in *E. coli* [152-155]. Later, Thomas Silhavy and colleagues identified the role of Cpx pathway in ECS responsiveness [156-158]. The Cpx pathway is widely seen among Gram-negative bacteria pathogens [159]. The Cpx pathway consists of a transmembrane sensor kinase CpxA and its cognate RR CpxR present in the cytoplasm [160, 161]. The three distinct functions of CpxA are known based almost entirely on studies in laboratory *E. coli* K-12; these are autophosphorylation, phosphorylation of CpxR and phosphatase activity of CpxR [162]. The Cpx TCRS responds to signals like alterations to the composition of IM, alkaline pH and accumulation of specific misfolded outer membrane proteins (OMPs) as occurs upon over-expression of new lipoprotein E (NlpE) in the bacterial periplasm [163-167]. In the absence of inducing signals, autophosphorylation of CpxA is inhibited by an interaction with the periplasmic protein CpxP [168]. In the presence of inducing signals, misfolded proteins sequester CpxP from CpxA towards DegP for degradation. DegP is a periplasmic protein that functions both as a protease and chaperone and is critical for protein quality control functions in the periplasm [169, 170]. Free CpxA becomes autophosphorylated at the conserved histidine residue at position 249. The Phosphoryl group is subsequently transferred from CpxA to CpxR, leading to the phosphorylation of CpxR at conserved aspartic acid residue at position 51 [162, 163]. Phosphorylated CpxR (CpxR~P) is the active isoform, which homodimerizes to regulate gene transcription both positively and negatively [164-166].
In *Y. pseudotuberculosis*, mutant strains accumulating active CpxR~P isoform has shown to upregulate genes like *cpxP*, *degP*, *ppiA* and their products are involved in protein quality control in the periplasm and thereby maintaining the structural integrity of the bacterial OM [171]. Concurrently, these same mutants show a strong tendency of negative transcriptional regulation of virulent genes like *inv*, *rovA* and *ysc-yop* T3SS genes [163, 171-173]. However, even though Cpx involvement in both virulence gene regulation and maintaining quality control in bacterial envelope is well established, there are many questions that are needed to be addressed to obtain a clear picture. These include the identity of the signals that induce Cpx pathway signalling during host infection, the molecular mechanisms behind the positive and negative regulation of gene expression by CpxR~P and the true mechanism of CpxR activation. CpxR~P mediated transcriptional regulation in *Y. pseudotuberculosis* is the main subject of this thesis and will be further discussed in the results and discussion.
Figure 6: Overview of Cpx pathway signalling. Under non-inducing condition (left side of the figure) autophosphorylation of CpxA is prevented by interaction of CpxP. Under inducing conditions (right side of the figure) the misfolded proteins in the periplasm titrates away CpxP towards DegP that leads to autophosphorylation of CpxA. Autophosphorylated CpxA donates its phosphoryl group to CpxR leading to the phosphorylation and dimerization of CpxR that transcriptionally regulate genes both positively and negatively.

1.9.1.1 Structural features of CpxA and CpxR

CpxA belongs to the class 1 sensor kinase family that has two transmembrane domains, a large periplasmic domain and a cytoplasmic domain having a conserved kinase core [174, 175]. The consecutive domains in the N-terminal domain are responsible for sensing and processing input signals [176]. The cytosolic domain harbors a HAMP domain that connects
the second transmembrane domain of CpxA with the kinase core and is assumed to act as a link that transmits signals from the sensor domain to the kinase core [175, 177]. The HAMP domain gets its name due to its presence in Histidine kinases, Adenyl cyclases, Methly-accepting proteins and Phosphatases [176]. The kinase core that functions as a transmitter domain consists of a dimerization histidine phosphotransfer (DHp) domain and a C-terminal catalytic ATP-binding (CA) domain. The DHp domain is involved in dimerization and possesses a conserved histidine residue and a phosphatase domain in order to dephosphorylate CpxR [178-180]. The C-terminal catalytic domain possesses a number of conserved motifs that are necessary for ATP binding [181, 182].

**Figure 7: Schematic diagram of CpxA domains:** Different domains of CpxA-N-terminal sensor input domain, HAMP domain, Dhp domain and C-terminal histidine kinase catalytic domain are represented by varying color codes. The domains of CpxA from *Y. pseudotuberculosis* was identified by means of alignment in protein family database (pfam).

CpxR is included in the large OmpR/PhoB family of winged-helix-turn-helix transcriptional response regulators (RRs) [183]. CpxR contains an N-terminal receiver domain with an aspartate residue (D51) that functions as the site of phosphorylation to promote dimerization along with a C-terminal effector domain that functions as a transcriptional regulator of target genes [175, 184, 185]. The two domains are joined by a flexible internal linker [186]. CpxR\(~\)P recognizes the DNA sequence 5’-GTAAA(n5)GTAAA-3’ as its
consensus binding box and binds to these regions by means of a winged-helix-turn-helix motif in the effector domain [187-189].

**Figure 8: Schematic diagram of CpxR domains:** Different domains of CpxR-the N-terminal domain, linker and C-terminal domains are represented by varying color codes. The figure is modified from [186].

### 1.9.2 RpoE/σ^E^ envelope stress response

The σ^E^ envelope stress response is a well studied ECS responsive pathway in *E. coli*. The σ^E^ pathway becomes activated due to osmotic and oxidative stress, accumulation of misfolded OMPs and heat shock [185]. This pathway consists of the RseA protein present in the IM of bacteria and this function as a specific anti-σ^E^ factor [190, 191]. The RseB protein located in the periplasm, binds to RseA to enhance the anti-σ^E^ activity of RseA [191]. The IM proteins DegS and RseP both function as proteases. On inducing conditions that constitute ECS, the C-terminus of unfolded OMPs interact with DegS to activate it [192-196]. The activated DegS cleaves off the periplasmic domain of RseA thereby sequestering RseB from RseA. This sets into motion a proteolytic chain of events whereby RseP cleaves off the transmembrane region of RseA releasing it into the cytoplasm. The cytoplasmic ClpXP protease then degrades the residual cytoplasmic–located RseA that permits the release of σ^E^ [197, 198]. Released σ^E^ is then free to bind core RNA polymerase that can then transcribe the genes involved in the composition and folding of proteins in the cell envelope in an effort to maintain the OM integrity [199]. In *E. coli*, maintaining OM integrity
includes $\sigma^E$ ensured porin production and LPS export into the OM [200-202].

In *Y. enterocolitica*, the *rpoE* gene is essential for its growth [203, 204]. Our own preliminary results observed upon removing the *rpoE* gene suggest that it might also be essential in *Y. pseudotuberculosis* [173]. Even though further proof is needed, *Y. pseudotuberculosis* lacking the *rseA* encoding anti-$\sigma^E$ showed a slight elevation of Ysc-Yop plasmid encoded T3SS in non-inducing conditions. This could indicate a link between the ECS response $\sigma^E$ pathway and T3SS [173, 205].

**Figure 9: RpoE ECS responsive pathway in Yersinia.** During inactive conditions RpoE is bound by anti-sigma factor RseA preventing its activation. Activation of RpoE pathway is followed by a series of proteolytic cleavages separating RpoE that influence T3SS with an unknown mechanism.
1.9.3 Phage shock protein pathway

The name phage shock was given to the pathway due to the overexpression of a particular component in *E. coli* infected with filamentous phage f1, and was specifically due to the phage gene protein pIV. The pIV protein is a secretin that is normally used for phage extrusion from the cell [206, 207]. The overexpressed bacterial protein was later renamed phage shock protein A (PspA) and it is encoded by the polycistronic operon *pspABCDE* [207]. PspB and PspC are located in the IM, as is PspA but only during inducing conditions [207, 208]. PspA functions as a negative regulator of Psp expression [209]. On the other hand, PspF is a transcriptional factor encoded divergently upstream of *pspA* operon and functions as a positive regulator of the *psp* operon. During non-inducing conditions PspA binds to PspF preventing its complex formation with σ54 on the RNA polymerase holoenzyme, which drives transcription from the *pspA* promoter [210, 211]. The Psp pathway is shown to be induced by extreme high temperatures, ethanol exposure and osmotic shock [207, 209]. Upon exposure to an inducing signal PspA is recruited into the IM where it interacts with PspB and PspC, releasing PspF to interact with ı54 to specifically upregulate the *pspA* operon [212, 213].

In *Y. enterocolitica*, a transposon insertion mutant in *pspC* was avirulent during intraperitoneal injection of mouse [214]. Most likely this is due to the Psp pathway being essential during T3SS inducing conditions. During inducing conditions that promote T3SS, the YscC secretin - an OM component of T3SS machinery can be mislocalized to the cytoplasmic membrane leading to compromised IM integrity in *Y. enterocolitica*. These mislocalized secretins induce Psp pathway signalling essential for their viability [215, 216]. Even though the mechanism is not known PspBC is responsible for preventing secretin induced toxicity in *Y. enterocolitica* [217-219].
**Figure 10: Psp pathway in Yersinia.** During non-inducing conditions (shown in the left side) PspA present in the cytoplasm binds to transcriptional regulator PspF and inhibits it. During inducing conditions (shown in the right side) the PspBC separates PspA from PspF. This leads to positive regulation of \( pspA \) operon by PspF.

### 1.9.4 RCS phosphorelay

Regulation of capsular protein synthesis (Rcs) phosphorelay system is a more complicated signaling pathway than the standard TCRS. It came into the lime light during the search for regulators of capsular polysaccharide synthesis (CPS) in *E. coli* [220]. Rcs ECS responsiveness is exclusively found in the Enterobacteriaceae family of Gram-negative bacteria [221]. The Rcs pathway consists of a hybrid IM sensor kinase RcsC containing both a transmitter histidine domain (H1) and a receiver aspartate domain (D1). The RR RcsB is located in the cytoplasm and functions as a transcription factor [222]. An additional protein, RcsD, is present in the IM and contains a
unique histidine-containing phosphotransfer (Hpt) domain [223]. Yet another component, RcsF, is an OM lipoprotein that is implicated to function as a sensory protein for several inducing cues [224, 225]. Under non-inducing conditions, the pathway is turned off by the phosphatase activity of RcsC directed at RcsB [185]. The inducing signals for Rcs includes over production of envelope proteins, desiccation, perturbations in extracellular polysaccharide production and osmotic shock [205, 221]. In one or more of these conditions, the stress signal enters the pathway either through RcsF or directly through RcsC leading to the autophosphorylation of RcsC at H1 domain and this will be transferred to D1 domain. The Hpt domain in RcsD mediates the transfer of this phosphoryl group from RcsC to RcsB [185]. Phosphorylated RcsB is active and causes diverse changes in bacterial physiology such as enhanced capsulation and decrease in flagellar biosynthesis [226] that can be helpful in survival against certain ECS.

In *Y. enterocolitica*, a chromosomally encoded *Yersinia* secretion apparatus - *Yersinia* secreted proteins (Ysa-Ysp) T3SS is controlled by the Rcs pathway [227]. Interestingly, recent findings showed that Rcs pathway activation can positively regulate the plasmid encoded Ysc-Yop T3SS in *Y. pseudotuberculosis* by RcsB-mediated increased transcription of *lcrF* the transcriptional activator of *ysc-yop* gene expression [228]. The *rcs* locus is present in all *Yersinia* species, but at the same time the *rcsD* is a natural pseudogene in *Y. pestis* [229]. The specific maintenance of Rcs phosphorelay pathway in enteric human pathogenic *Yersinia* might therefore be related to the route of infection, since an intact Rcs pathway was shown to be necessary for successful oral infections of mice with *Y. enterocolitica* [227].
Figure 11: Rcs phosphorelay system in Yersinia. In the absence of inducing signals in Rcs phosphorelay system (shown in the left side), RcsC functions as a phosphatase for RcsB. Activation of Rcs phosphorelay system (shown in the right side) phosphorylates RcsB leading to the increased expression of T3SS master transcriptional regulator lcrF and thereby T3SS.
2. Objectives of this thesis

The main aim of this thesis was to investigate the regulatory control by the CpxRA TCRS of virulence-associated functions in *Y. pseudotuberculosis*.

Specific aims:

a) To determine the involvement of CpxR~P in negative transcriptional regulation of *rovA* and uncover the molecular mechanism

b) Determine if CpxR~P can control *rovA* expression by controlling RovM levels.

c) To define the contribution of accumulated CpxR~P in controlling Ysc-Yop T3SS

d) To develop assays to analyze CpxR homodimerization and the molecular interaction between CpxA and CpxR
3. Results and discussion

3.1 Regulation of *rovA* and *rovM* by accumulated CpxR~P

The Cpx pathway is an ECS responsive pathway that can become activated on encountering ECS. Cpx TCRS consists of the CpxA sensor kinase and the cognate response regulator CpxR. It is well known that an activated Cpx pathway leads to phosphorylation of response regulator CpxR that is involved in both positive and negative transcriptional regulation of genes alleviating the after effects of ECS (see section 3.1 for more details on Cpx pathway signalling). In *E. coli*, the CpxA sensor kinase has been shown to have 3 distinct functions - autophosphorylation, phosphorylation of CpxR and phosphatase activity of CpxR (figure 12) [162]. It follows that CpxR~P accumulates on generation of CpxA phosphatase defective mutant strains such as by the full-length deletion of the *cpxA* allele - termed Δ*cpxA* null strain, and the gain of function mutant with an allelic variant CpxA*T253P* that is routinely designated as *cpxA*101* [156, 157, 162, 230-232]. A link between the Cpx pathway and pathogenesis in *Y. pseudotuberculosis* was shown in our group. The removal of *cpxA* from *Yersinia* caused a down regulation of virulent factors like T3SS, RovA and invasin, which have all been shown to be important for virulence during mouse infection studies [171, 173] (More details on T3SS, RovA and invasin are given in section 1.7.4, 2.1 and 1.7.1 respectively). Specifically, the contribution of accumulated CpxR~P in repressing expression of *rovA* and the mediator role played by RovM that functions as a negative transcriptional regulator of *rovA* are discussed in papers I and II respectively.

3.1.1 *In vitro* phosphorylation of CpxR and DNA-binding to the promoter regions of *rovA* and *inv*

It is speculated that phosphorylation of the response regulator stimulates structural changes that lead to the formation of functional homo dimers that is an important prerequisite for efficient binding of target DNA.
Acetyl phosphate (acetyl-P) was used as phosphor donor for in vitro phosphorylation of purified CpxR. Based on the homology with OmpR/PhoB family of response regulators, a conserved aspartate site at position 51 (Asp$_{51}$) in CpxR was predicted to be the phosphorylating site in CpxR [233]. Using an In vitro phospho protein gel staining assay, phosphorylation of wild type CpxR, but not with a substitution of Asp$_{51}$ with alanine, could be demonstrated (paper 1, figure 2). Even though CpxR$_{D51A}$ was not phosphorylated during in vitro assays, there is still a possibility that the substitution can cause an allosteric effect that can prevent phosphorylation at an alternative site.

In vitro assay such as electrophoretic mobility shift assay (EMSA) was used to show direct binding of phosphorylated CpxR on PCR amplified regions of promoters driving the expression of genes encoding for the virulence factors, RovA and invasin. At the same time CpxR$_{D51A}$ was unable to bind on those promoters, indicating that under these experimental condition phosphorylation might be necessary for DNA-binding (paper 1, figure 3). However, this is likely to be assay dependent because our group had earlier shown that non-phosphorylated CpxR could bind slightly to these same promoter regions [171]. This makes it difficult to conclude whether CpxR needs to be phosphorylated for DNA-binding. Further biochemical studies are needed to address this crucial point.

### 3.1.2 Accumulation of CpxR~P in ΔcpxA and cpxA101*

In *Y. pseudotuberculosis*, ΔcpxA and cpxA101* accumulates two different isoforms of CpxR - phosphorylated CpxR (active form) and non-phosphorylated CpxR (inactive form). In the absence of cognate CpxA, it is speculated that accumulation of CpxR~P is mediated through acetyl~P and other small molecular phospho-donors that are metabolic intermediates. The phosphor-donor acetyl~P is derived from phosphotransacetylase (Pta) – acetate kinase (AckA) pathway [234]. Phos-tag acrylamide methodology along with immunoblotting was utilized to analyze and distinguish these two
isoforms of CpxR. An analysis of isoforms produced by the ΔcpxA and cpxA101* strains growing under different nutrient conditions like lysogeny broth (LB) media (paper II, figure 2A) and Roswell Park Memorial Institute (RPMI) medium (paper II, figure 2B) revealed that the pool of total isoforms present was not affected. However, the ΔcpxA mutant routinely produced a higher pool of isoforms in all media conditions, when compared to the mutant strain cpxA101*. Crucially, deprivation of nutrients favored accumulation of CpxR~P (paper II, comparison between figures 2A and 2B).

![Figure 12: Distinct functions of CpxA sensor kinase – autophosphorylation, phosphorylation of CpxR and phosphatase activity of CpxA.](image)

3.1.3 DNA-binding site identification of CpxR~P on the rovA promoter region and its necessity for repressing rovA transcription

The transcription of rovA occurs from two different promoters termed P1 and P2 that are located upstream of the translational start site (a detailed description about RovA can be found in section 2.1). A DNA footprinting
assay showed that CpxR~P can bind to the sequence 5’-ACAAA(N)5ACAAA-3’ that overlaps with the -35 region at the P2 promoter of rovA that is located around 360 nucleotides upstream of the translational start site (paper 1, figure 4). An EMSA was conducted on template DNA containing the scrambled mutations Mt 1 (-35 box sequence of the P2 promoter is altered) and Mt 2 (unaltered -35 box but neighboring sequence altered) at the CpxR~P binding site in the rovA promoter region. This showed that CpxR~P can bind on PCR amplified DNA with Mt 1 background, but not with Mt 2 (paper 1, figure 5B). In order to know the in vivo effects of these shuffled mutations, they were introduced in cis into the chromosome of cpxA101* making cpxA101*, rovA (Mt 1) and cpxA101*, rovA (Mt 2). Immunoblotting showed some restoration (although not complete) of RovA production in strain cpxA101*, rovA (Mt 2) despite the presence of active CpxR~P (paper 1, figure 9), and this was correlated to semi-quantitative RT-PCR (paper 1, figure S3). Based on the results we speculated that CpxR~P binding to the -35 region of P2 promoter might occlude the positioning of RNA polymerase holoenzyme and/or formation of open promoter complex, thereby repressing rovA transcription.

3.1.4 Overexpression of NlpE reduced RovA and invasin expression

Overexpression of the OM protein NlpE has shown to activate Cpx pathway signalling in E. coli [158]. Since accumulation of CpxR~P using ΔcpxA and cpxA101* is a consequence of a short-circuit in the Cpx signaling pathway (i.e.: loss of CpxA phosphatase activity), NlpE from Y. pseudotuberculosis was over expressed in parental bacteria to examine the effects of activated Cpx pathway on RovA and invasin production when the signal transduction cascade remains intact. NlpE obtained from E. coli shows 50% identity at the amino acid level to NlpE from Y. pseudotuberculosis (paper 1, figure 8A). NlpE from E. coli and NlpE from Y. pseudotuberculosis were overexpressed in both the parent strain and the ΔcpxR null mutant. Immunoblotting results of the whole cell lysates indicate
the repression of RovA and invasin in the parental strain expressing NlpE from *E. coli* as well as *Y. pseudotuberculosis* (paper 1, figure 8B). The greater repression of RovA and invasin by NlpE from *Y. pseudotuberculosis* in the parent strain might be helpful in finding the mechanism behind the NlpE-dependent activation of Cpx pathway signalling. Repression of RovA and invasin was not observed in strains lacking CpxR proving that this repression requires CpxR (paper 1, figure 8B).

### 3.1.5 Increased expression of RovM in CpxA phosphatase defective mutants

RovM functions as a negative transcriptional regulator of *rovA*, and an increase in expression of RovM is observed during growth in minimal media [104] (a detailed description about RovM can be found in section 2.4). Using immunoblotting, enhanced expression of RovM was observed in the Δ*cpxA* and *cpxA101*+ mutants (paper II, figure 1), and this corroborated RT-PCR data with same strains growing in minimal media (paper II, figure 3). Comparison of RovM expression between parent and Δ*cpxR* strain showed the requirement for intact CpxR to increase expression of RovM when grown in minimal media (paper II, figure 1B). Thus, accumulated CpxR~P was shown to be repressing *rovA* through two modes one by direct repression and the other indirectly through enhanced transcription of *rovM*.

### 3.1.6 Mapping of the CpxR~P DNA-binding site within the *rovM* promoter region and its impact on the regulation of *rovM* expression levels

A DNA-footprinting assay showed that CpxR~P can bind at two positions termed box 1 and box 2 within the *rovM* promoter (paper II, figure 4A). Box1 is located in the non-coding reverse strand and box 2 is located in the coding forward strand in the promoter region of *rovM* (paper II, figure 4A). Interpretation of DNA-footprinting results indicated box 1 to have higher affinity for CpxR~P in comparison with box2. Scrambled mutations to
disrupt both box 1 and box 2 CpxR~P binding sites in rovM promoter region were generated in the CpxA phosphatase defective mutants backgrounds – termed ΔcpxA, rovM (Mt.1), cpxA101*, rovM (Mt.1), ΔcpxA, rovM (Mt.2) and cpxA101*, rovM (Mt.2) (paper II, figure 4). Corroborating footprinting data, immunoblotting results showed box 1 being primarily responsible for CpxR~P dependent expression of RovM (paper II, figure 5). The biological relevance of box 2 is difficult to define.

3.1.7 Presence of RovM and CpxR~P are required for transcriptional silencing of rovA

Transcription of rovA is repressed by two mechanisms, first by co-operative binding between H-NS and RovM [104] and second by means of accumulated CpxR~P in Y. pseudotuberculosis (paper I). Direct repression of rovA mediated through accumulated CpxR~P was revealed in phosphatase defective mutants lacking RovM (paper II, figure 6). This showed the dominance of RovM mediated repression over CpxR~P mediated repression. At the same time however, it is possible that co-operational binding between RovM and CpxR~P might be needed for CpxR~P to maximize its ability to repress rovA expression.

Phosphatase defective CpxA mutant strain that also had a scrambled mutation of the rovA promoter that prevented CpxR~P binding, incompletely restored expression of RovA (paper 1, figure 9). The incomplete restoration of RovA was speculated to be due to the repressive effect of RovM that was still active. To overcome this, a third mutation was introduced to alter the rovM promoter so that it was also unable to be bound by CpxR~P. This new strain accumulates CpxR~P, but neither the rovA nor the rovM promoters are responsive to it. An immunoblot of total protein showed fully restored expression of RovA (paper II, figure 7). Hence the silencing of rovA is mediated by both RovM and CpxR~P, albeit to different degrees with RovM being the primary repressor.
A reduction in virulence caused by a decreased adherence to host cells mediated through active Cpx pathway signalling has already been observed in *Shigella* spp. [235] as well as *Salmonella enterica* serovar Typhimurium [236]. CpxR~P has shown to repress *rovA* as well as *inv* and positively regulate expression of *rovM* by direct binding on their promoter region. It is interesting to know why CpxR~P needs to regulate *inv* mediated through *rovA* as well as *rovM*. Increased expression of RovM has shown to be hypermotile in *Y. pseudotuberculosis* [104], this might be helpful for the bacteria to escape from a certain ECS. Moreover, it is possible that the energy saved by preventing virulence can be useful for the bacteria to better cope with the effects of ECS to enhance bacterial survival.

### 3.2 Accumulated CpxR~P mediated repression of T3SS

The CpxA phosphatase defective mutants have already been shown to repress Yops synthesis and secretion during T3SS inducing conditions [173]. Contribution of accumulated CpxR~P in the repression of T3SS is addressed in paper III.

#### 3.2.1 Accumulation of CpxR~P in CpxA phosphatase defective mutants

The CpxA phosphatase defective strains were grown in T3SS inducing conditions (paper III, see materials and methods section 2.4) in brain-heart infusion (BHI) and LB media. Different isoforms of CpxR were differentiated by means of fractionation on a phos-tag gel and then immunoblotting. Accumulation of CpxR~P was observed in both Δ*cpxA* and *cpxA101* strains irrespective of the growth medium (paper III, figure 3). These results strengthened the idea that repression of T3SS was mediated through accumulated CpxR~P. Accumulated CpxR~P mediated repression of T3SS requires presence of functional CpxR (paper III, figure 4C).
3.2.2 CpxR~P binds directly to the regulatory regions of genes encoding key T3SS regulators

An EMSA was used to demonstrate binding of CpxR~P on gene promoters encoding various T3SS components including polycistronic operons yscA-M and yscN-U (both encode structural components of T3SS), yopNtyeAsycNyscXY (encoding regulatory and structural components) and lcrGVHyopBD (encoding translocators and regulatory components). The monocistronic operons used in EMSA includes lcrF and lcrQ (encoding regulators), yopE, yopH and yopK (all encoding translocated anti-host effectors) and sycE and sycH (encoding T3SS chaperones of YopE and YopH). High affinity binding of CpxR~P was observed on promoters of lcrF and yopK and medium affinity binding was observed on yopH, yopE, sycH and lcrQ promoters. Finally, CpxR~P binding was not observed on promoters of yopN, lcrG, yscA and yscN (paper III, figure 6).

Based on the results obtained from DNA-binding assays there are a number of different possibilities for CpxR~P mediated repression of T3SS. All these need to be explored in the future. Based on the EMSA results that show strong binding between CpxR~P and promoter region of T3SS master regulator lcrF, CpxR~P-dependent control of lcrF transcription is a likely scenario. However, the gene expression of lcrF in ΔcpxA did not show a significant difference to parental bacteria [173]. At the same time, gene expression of yopE and yopK was significantly reduced [173]. Thus, the suppression of T3SS by repressing anti-host effectors might be one of the mechanisms by which CpxR~P might be suppressing T3SS activity. Host cell attachment of bacteria is an important step in effective T3SS-dependent Yop delivery [92]. Crucially, removal of cpxA gene decreases the expression of invasin adhesin [171]. This could mean that T3SS can be repressed due to defective host cell contact, which represents another possibility for CpxR~P mediated repression of T3SS. Yet another possibility of repressing T3SS can be envisioned by increasing the expression of lcrQ that encodes a negative regulator of T3SS [92, 98, 99]. Additionally, YtxR is a LysR type
transcriptional regulator that functions as a transcriptional off switch for T3SS in *Y. enterocolitica* [237]. YtxR is anticipated to be in competition with LcrF in order to bind on overlapping DNA sequences within the *ysc-yop* promoter regions. However, the environmental signals that control the function of YtxR are not known. Our EMSA result shows comparatively strong binding by CpxR~P in the promoter region of *ytxR* (Thanikkal EJ, unpublished) showing that another possible way of repressing T3SS could be by increasing the expression of YtxR. Different predicted transcriptional regulations mediated through CpxR~P based on the results of DNA-binding assays are shown in figure 13.

It is also a possibility that partitioning control of the virulence plasmid harbouring the *ysc-yop* genes is compromised by elevated CpxR~P levels. The *sopABC* locus is known to ensure plasmid partitioning [238], our EMSA result shows strong binding by CpxR~P in the promoter region of *sopA* (Thanikkal EJ, unpublished) showing another possibility for repressing T3SS. The periplasmic protein DegP is known to get induced during activated Cpx pathway signaling [157]. Recent results show that DegP is involved in posttranscriptional regulation of the T3SS in enteropathogenic *E. coli* [239] showing yet another possibility of repressing T3SS.
Figure 13: Predicted transcriptional regulation by CpxR~P. The transcriptional regulation in *Y. pseudotuberculosis* by CpxR~P on different virulent gene promoters are represented. The green colored lines indicate positive regulation and red colored lines indicate negative regulation.

### 3.3 Analysis of the interaction between CpxA and CpxR using hybrid-based protein-protein interaction assays

TCRS’s play significant roles in regulating physiological processes in bacteria by means of transcription, post transcription and post translation. Molecular interactions like protein-protein interaction and protein-DNA interactions are exploited by TCRS during their regulatory processes [174, 240, 241]. In paper IV, on analysis of homodimerization of CpxA and CpxR, as well as the interaction between CpxA and CpxR was initiated by means of a bacterial adenylate cyclase two hybrid (BACTH) assay and a λcI homodimerization assay that check the *in vivo* protein-protein interaction potential.
3.3.1 BACTH assay

The CpxA sensor kinase is known to form a homo dimer [175, 240]. BACTH analysis further showed the dimer formation of CpxA from Y. pseudotuberculosis (paper IV, figure 1). Moreover, an assay to measure CpxA-CpxR interaction indicated that full length CpxA can interact with CpxR provided that CpxR contains an intact and functional N-terminal domain (paper IV, figure 2). This supports in silico predictions showing cross talk between N-terminal domain of CpxR with CpxA [242, 243]. A BACTH analysis of the CpxR-CpxR interaction shows that the N-terminal domain of CpxR plays a significant role in homodimerization, which is enhanced by the presence of the internal linker domain (paper IV, figure 5). Even though we could not confirm the stability of in vivo protein expression in the assay, results of BACTH assay support the previous findings that head-to-head symmetrical dimers mediated by $\alpha_4-\beta_5-\alpha_5$ interface present in the N-terminus of response regulators was conserved in OmpR-PhoB family [240, 244]. However, to confirm this, a crystal structure of CpxR in both active and inactive form would be desirable.

3.3.2 λcI homodimerization assay

The λcI homodimerization assay data demonstrated dimerization of full length CpxR, and indicated that this was dependent on the presence of the C-terminal domain, which was enough for dimerization (paper IV, figure 7A). Frustratingly, this was completely opposite to what was observed in the BACTH assay. However, trans-expression of just the C-terminal domain alone is constitutively active in some assay conditions leading to the transcription of target genes in E. coli [186]. In addition to target DNA, the CpxR C-terminal domain is suppose to interact with one of the six subunits of RNA polymerase holoenzyme. Although this is well established for some other response regulators like PhoB that interacts with $\sigma^{70}$ subunit [245-247] and OmpR that interacts with the $\alpha$ subunit [248, 249], it is still not known
for CpxR. Nevertheless, it indicates the possibility of the CpxR C-terminus alone being able to dimerize as an active entity that can also engage with DNA and the holoenzyme of RNA polymerase based on our results.

Even though many of the results from the BACTH and λcI homodimerization assays seem to be contradictory, they were always reproducible. Therefore, the BACTH assay can ideally be used in analyzing CpxR interactions involving its N-terminus while the λcI homodimerization assay can aid in analyzing C-terminal interactions between CpxR monomers.
Main findings in this thesis

- Accumulated CpxR~P can directly repress the transcription of *rovA* and *inv*.

- Accumulated CpxR~P can directly enhance expression of *rovM*.

- CpxR~P acts by binding to regulatory regions within the promoters of the *rovM*, *rovA* and *inv* genes.

- CpxR~P mediated repression of *rovA* is secondary to the effect of RovM.

- Repression of T3SS in CpxA phosphatase defective mutants is mediated by accumulated CpxR~P.

- BACTH assay can be useful in analyzing molecular interactions involving the N-terminal domain of CpxR.

- λcl homodimerization assay can be useful in analyzing molecular interactions involving the C-terminal domain of CpxR.
Future perspectives

This thesis is mainly concentrated on defining the involvement of accumulated CpxR-P in regulating expression of virulence factors like inv, rovA, rovM and T3SS that are significant in Y. pseudotuberculosis pathogenesis. Even though the involvement of Cpx TCRS in virulence regulation was known, the mechanism behind how accumulated CpxR-P can promote positive regulation of one gene and repress another is needed to be explored.

Our studies showed that accumulated CpxR-P can repress rovA expression and enhance the expression of its negative regulator rovM by direct binding to its promoter region. In order to find out the mechanism behind how accumulated CpxR-P regulate genes both positively and negatively, we have to make various CpxR derivatives that are (1) defective in DNA-binding (2) constitutively active or constitutively inactive and (3) defective in inter-molecular or intra-molecular interactions. BACTH and λCI homodimerization assay can be helpful in analyzing phenotypes of such CpxR derivatives based on the location of the mutation and its expected phenotypic property. Protein-protein interaction assays to find out the RNA polymerase subunit that binds on CpxR during its gene regulation are imperative. In the long run, an in vitro transcription assay would need to be conducted to find out the dual regulation mechanism followed by CpxR-P.

This thesis presents data showing the involvement of CpxR-P in preventing T3SS by means of an unknown mechanism. CpxR-P was shown to bind on promoters of different substrates of T3SS. Thus, an initial plan would be to mutate the binding box of CpxR-P within these promoters based on their regulatory hierarchy and check whether T3SS is still repressed. Finally, an in vitro transcription assay can be developed to find the exact mechanism behind repression of T3SS mediated through accumulated CpxR-P.

Most of the studies performed here are on activated Cpx pathways that have been generated artificially by mutation. It is of interest to perform assays where activation of Cpx pathway is exclusively happening only due to
ECS signals i.e. where the pathway is genetically intact. Finally, long term goal is to develop novel antimicrobial drugs that prevent bacterial pathogenicity in *Yersinia* by turning on Cpx pathway. A chemical high throughput screen could be developed on a strategy of bacteria having a stable reporter construct that can sense accumulated CpxR–P.
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