STUDY OF REGULATORS AFFECTING TYPE III AND TYPE VI SECRETION SYSTEMS IN *EDWARDSIELLA TARDA*

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LIST OF PUBLICATIONS RELATED TO THIS STUDY

1. **Chakraborty S,** Mo L, Chatterjee C, Sivaraman J, Leung KY, Mok YK (2010)Temperature sensing and regulation of virulence by a novel PhoP-PhoQ two-component system in *Edwardsiella tarda*. *J.Biol.Chem* **285 (50)**:38876-88

2. **Chakraborty S**, Chatterjee C, Sivaraman J, Leung KY, Mok YK: Crosstalk between Phosphate and Iron mediated Regulation of Type III and Type VI secretion system in *E. tarda* Manuscript in preparation.

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LIST OF ABBREVIATIONS

aa	Aminoacid
Amp^r	ampicillin-resistant
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pairs
BSA	bovine serum albumin
6-FAM	6 – Carboxyfluorescein
Col^r	colistin- resistant
CFU	colony forming umits
Cm	centimeter(s)
ºC	degree Celsius
DBD	DNA binding domain
DNA	deoxyribonucleic acid
ECP	extracellular protein
EDTA	ethelyne diamine tetra acetic acid
EPC	epitelioma papillosum of carp, *Cyprinus carpio*
EVP	*E. tarda* Virulence protein
g	Gram
g	gravitational force
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IPTG	isopropyl-thiogalactoside
kb	kilo base
Kan^r	kanamycin-resistant
l	litre(s)
LB	luria-Bertani broth
LBA	luria-Bertani agar
M	molarity, moles/dm^3

mg	milligram(s)
min	Minute
ml	milliliter(s)
mM	milli moles/dm^3
Neo^r	neomycin-resistant
orf	open reading frame
OD	optical density
%	Percentage
PAGE	poly acrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Pi	in-organic phosphate
PPD	primary Production Department
ppm	parts per million
PST	Phosphate specific transport
ROI	reactive oxygen intermediates
RE	restriction enzyme
SDS	sodium dodocyl sulfate
SPI-1	*Salmonella* pathogenecity island 1
SPI-2	*Salmonella* pathogenecity island 2
SOD	super oxide desmutase
TE	tris- EDTA
Tn*phoA*	transposon carrying promoter-less alkaline phosphatase
TSA	tryptic soy agar
TSB	tryptic soy broth
T3SS	type III secretion system
T6SS	type VI secretion system
TRs	transcriptional regulators
U	unit(s)
µg	microgram(s)

µl	microlitre(s)
wt	wild type
v/v	volume per volume
w/v	weight per volume
X-gal	5- bromo-4-chloro-3-indolyl-B-D-galactopyranoside

SUMMARY

Edwardsiella tarda is an opportunistic gram-negative bacterial pathogen possessing multifactorial virulence determinants such as abilities to invade epithelial cells, resist phagocytic killing and produce hemolysins and catalases. Type III and type VI gene clusters have been identified which play a pivotal role in the pathogenesis of *E. tarda*. Cross-talk between different regulatory systems in pathogenic bacteria is important for systematic cell to cell communication. Multiple signaling molecules are frequently required to trigger differential responses in bacteria in the quest for survival inside host cells. In this study, we have demonstrated activation of virulence genes in *E. tarda* in response to various environmental signals that mimic conditions inside host cells. *E. tarda* shows a unique temperature dependent secretion profile in which temperature ranges from 23°C to 35°C enable the bacteria to secrete virulence proteins. A decrease in temperature from 23°C to 20°C or an increase in temperature from 35°C to 37°C completely abrogates the secretion of virulence proteins. We have identified the two-component regulatory system, PhoP/PhoQ which regulates positively positively both the type III and type VI secretion systems as mutation of both *phoP* and *phoQ* abolished protein secretion from T3SS and T6SS. The global response regulator PhoP directly binds to a putative PhoP box present in the promoter of *esrB* which is a regulator present within the T3SS gene cluster. The expression of both *phoP* and *esrB* is temperature dependent where the transcriptional levels of both *phoP* and *esrB* are higher at temperatures from 23°C to

35°C but decreased significantly at 20°C and 37°C in agreement with the secretion profile. Apart from temperature, *E. tarda* also responds to Mg^{2+} in which low Mg^{2+} concentration (1mM) triggers the expression of both *phoP* and *esrB* along with secretion of type III and type VI proteins, while Mg^{2+} (10mM) inhibits expression and secretion of protein from both T3SS and T6SS. The change in environmental temperatures is sensed by the PhoQ protein. The periplasmic sensor domain of PhoQ shows loss of secondary structure when the temperature is increased from 35°C to 37°C with an exceptionally low Tm at 37°C based on temperature denaturation experiments using Far-UV CD experiments. Addition of Mg^{2+} slightly stabilized the sensor domain and and Tm is shifted to 40.2°C. Using site-directed mutagenesis technique we have identified certain Pro and Thr residues in *E. tarda* PhoQ sensor domain that are responsible for the low temperature stability of PhoQ. PhoQ mutants P120N (Tm=55.5^{o}C) and T167P (Tm=59.0^{o}C) showed significant higher thermal stabilities than the wild type protein. Complementation of the *phoQ* insertion mutant (*phoQ*$_{i}$) with the above mutants rendered "loss-of-function" phenomena where the mutants failed to recover the effect of the *phoQ*$_{i}$ mutation. Interestingly, the mutant P77L rendered the *E. tarda phoQ*$_{i}$ strain "temperature-blind" which resulted in the constitutive secretion of proteins from T3SS and T6SS at 20^{o}C but not at 37 ^{o}C. Moreover, *E. tarda phoQ*$_{i}$ mutant, when complemented with *phoQ* from EPEC 2348/69 showed similar levels secretion of ECPs at 37^{o}C as compared to the wild type *E. tarda* at 30^{o}C. We also identified acidic cluster residues (DDDSAD) present within the sensor domain of PhoQ that are responsible for Mg^{2+} binding. Based on the two-dimensional electrophoresis profile of the total cell protein and microscopic examination of *E. tarda* at different temperatures, different regulatory mechanisms could be employed at 20 ^{o}C and 37 ^{o}C.

We further extended our study on the regulation of *E. tarda* by two environmental factors, iron and phosphate, since such environmental cues are also sensed inside host cells. Both high phosphate (20mM KH_2PO_4) and high iron (20μM $FeSO_4$) decresed the secretion and expression of T3SS and T6SS proteins by modulating the expression of *esrC* where greater effect was observed in presence of iron in comparison to that of phosphate. We have characterized the iron sensor Fur to be a negative regulator of T3SS and T6SS which functions through *esrC*.We identified the presence of a high affinity PhoB binding site (pho box) in the promoter region of *evpA* within the T6SS cluster which allows PhoB to positively regulate the expression of *evpC* of T6SS. PhoB binds to the polycistronic promoter of *pstSCAB-phoU* operon and to its own promoter rendering a self regulatory function. The two component regulatory system PhoB/R responds to phosphate concentrations since a deletion mutant of *phoB* (*ΔphoB*) renders the bacteria to become "phosphate- blind" but still responsive to the suppressing effect of Fe^{2+}. PhoB functions by modulating the expression of *esrC* and the transcriptional level of *esrB* seems to be unaffected by both iron and phosphate. PhoU positively controls the expression and secretion of T3SS and T6SS proteins through *esrC* and may be involved in additional regulatory functions since two-dimensional PAGE analysis of the total protein of the insertion mutant of phoU (*phoU*$_i$) shows suppression in the expression of many proteins in comparison to the wild type bacteria. Fe^{2+} itself exhibits an inhibitory effect on the expression of *phoB* and the *pstSCAB-phoU* operon.Our study proposes a model where negative cross-talk exists between the high affinity phosphate transporter PstSCAB-phoU and the iron sensor Fur.

Chapter I. Introduction

I.1 *E. tarda* infection and virulence factors

I.1.1 Taxonomy identification and distribution

E. tarda belongs to the Enterobacteriaceae family and the genus *Edwardsiella* has two other species namely *E. hoshinae* and *E. ictaluri* in which *E. ictaluri* isolated from catfish was found to cause severe infections and enteric septicemia (Hawke et al, 1981); whereas *E. hoshinae* has been found in water, birds, and lizards (Grimont et al, 1980). *E. tarda* was formerly known as *Paracolobactrum anguillimortiforum,* which is presently, considered to be synonymous with *E. tarda* (Hoshina, 1962). Members of the *Edwardsiella* genus have been associated with freshwater and marine environments as well as with the animals residing in these ecosystems. *E. tarda* has a wide host range and geographical distribution compared to the other two species. The presence of *E. tarda* has been reported in India (Bhat et al, 1967), Malaysia (Gilman et al, 1971), Israel (Sechter et al, 1983), Japan (Onogawa et al, 1976), Panama (Kourany et al, 1977) and the United States (Desenclos et al, 1990). Though *E. tarda* occurs globally, it is more commonly found in tropical and sub-tropical regions. *E. tarda* is a gram-negative, facultatively anaerobic, rod-shaped (1 μm diameter and 3-4 μm long) and motile bacterium having peritrichous flagella. It is oxidase negative and ferments glucose under aerobic and anaerobic conditions and has little biochemical variability (Waltman & Shotts, 1986). The bacterium cannot utilize many sugars, hence the epithet *tarda* which means inactivity. In general, *E. tarda* can grow on TSA medium wherein they form small, round (0.5-2.0 mm diameter), raised and transparent colonies in 48 hours of incubation at 24-26°C (Meyer & Bullock, 1973). It can be identified either by using commercial kits such as API 20E (Sakai et al, 1993) BBL Crystal

12A and 12B strips. Rapid identification of *E. tarda* is possible through the use of PCR-based identification systems (Chen & Lai, 1998). It is uncertain if this bacterium is a primary or opportunistic fish pathogen, as it may form part of the normal microflora of fish surfaces (Wyatt et al, 1979). However, some evidence does exist that *E. tarda* was found in water within the vicinity of fish farms. It has been frequently isolated from lower and higher vertebrates such as fish (striped bass, largemouth bass, tilapias and carp) (Baya et al, 1997; Francis-Floyd et al, 1993) and amphibians (frogs and toads) (Mauel et al, 2002).

I.1.2 *E. tarda* infection

I.1.2.1 Infection in human

E. tarda is the only species of this genus that can infect humans and cause diseases in humans. Association of *E. tarda* with human diseases was first reported in 1969 (Jordan & Hadley, 1969). So far, at least 300 clinic cases have been reported. *E. tarda* infections in humans are more common in tropical and subtropical regions (Kourany et al, 1977), and in persons with exposure to the aquatic environments or exotic animals including amphibians and reptiles, and conditions leading to iron overload and dietary habits like ingestion of raw fish (Janda & Abbott, 1993; Wu et al, 1995). The diseases caused by *E. tarda* in human infections can generally be divided into two categories, namely, gastro- and extra-intestinal infections. Gastro-intestinal infections are common compared to extra-intestinal infections. Although infections caused by *E. tarda* in humans are uncommon, gastro-intestinal infections are very serious and the mortality rate reached up to 50% (Janda & Abbott, 1993). Some of the clinical symptoms of gastroenteritis caused by this pathogen are acute secretory enteritis, and intermittent watery diarrhea with mild fever (39-38.5°C). More severe forms of gastroenteritis

similar to enterocolitis have also been reported (Nagel et al, 1982; Ovartlarnporn et al, 1986). *E. tarda* has also been found to cause extra-intestinal diseases such as myonecrosis (Slaven et al, 2001), peritonitis with sepsis (Clarridge et al, 1980), septic arthritis (Osiri et al, 1997), bacteremia (Yang and Wang, 1999) and wound infections (Ashford et al, 1998; Banks, 1992).

I.1.2.2 Infection in animals

E. tarda has been isolated from a variety of higher animals including mammals, birds, reptiles and fish. *E. tarda* has caused diseases in cattle (Cenci et al, 1977), swine (Owens et al, 1974). Florida manatee (Forrester et al, 1975), monkeys (Kourany et al, 1977), dogs (van Assche, 1991), rock hopper penguins (Cook & Tappe, 1985) and wild vultures (Winsor et al, 1981) *E. tarda* is the etiological agent of *Edwardsiella* septicemia in fish, often referred to as "Edwardsiellosis" which is a mild to severe systemic disease primarily affecting warm water fish in the United States and Asia. *E. tarda* is primarily known to cause disease in Japanese eel (*Anguilla japonica*) and channel catfish (*Ictalurus punctatus*), leading to great losses in aquaculture every year in the United States and Asia (Janda & Abbott, 1993). Therefore, it is important to study the pathogenesis of *E. tarda* and find suitable strategies to either prevent or cure its infection.

I.1.3. Antimicrobial susceptibility, treatment and vaccination

Several investigations have been carried out to specifically address the *in vitro* susceptibility of *E. tarda* to antimicrobial agents. These include β-lactam antibiotics, cephalosporins, aminoglycosides, and oxyquinolones. Major resistance has been noted only with colistin and polymixin. Stock & Wiedemann studied the natural antibiotic susceptibilities of 102 strains of *Edwardsiella* species (42 strains of *E. tarda*, 41 strains of *E. ictaluri* and 19 strains of *E. hoshinae*) to 71 antibiotics. They found that all *Edwardsiellae* were naturally

sensitive to tetracyclines, aminoglycosides, most β-lactams, quinolones, antifolates, chloramphenicol, nitrofurantoin, and fosfomycin. *Edwardsiella* species were naturally resistant to macrolides, lincosamides, streptogramins, glycopeptides, rifampin, fusidic acid, and oxacillin (Stock & Wiedemann, 2001). Gastro-intestinal infections often do not require treatment since these illnesses resolve spontaneously without antibiotic treatment (Janda & Abbott, 1993). In some aggressive infections, amoxicillin and comitrazole have been used with success. Drugs such as cephalosporins and oxyquinolones have also shown good results based on *in vitro* studies. The development of vaccines against *E. tarda* infection is pursued in Japan and Taiwan. Vaccine preparations involved the use of whole cells, disrupted cells and cell extracts as immunogens (Salati et al, 1987a; Salati et al, 1987b; Salati & Kusuda, 1985; Salati & Kusuda, 1986; Tu & Kawai, 1999). There were discrepancies in the experimental trials which suggested further studies to thoroughly test the efficacies of different methods and types of vaccination protocols in *E. tarda*. They also clearly indicate the necessity for further understanding of the virulence genes and strategies used by this pathogen to cause disease. This will enable us to develop of a more suitable, efficient and protective vaccine against this pathogen.

I.1.4 Virulence factors of *E. tarda*

I.1.4.1 Adherence to host cells and invasion

The ability to adhere and invade is an important prerequisite for the establishment of foothold in a variety of host tissues, thus leading to successful colonization (Atkinson & Trust, 1980;Edelman et al, 2003). *E. tarda* is also known to have this capability (Janda et al, 1991) and exhibits two types of hemagglutinations, one of which was inhibited by mannose (MSHA), while the other was blocked by glycoprotein fetuinn (MRHA) (Wong et al, 1989).

Both the MSHA and MRHA adhesions were resistant to mechanical shearing and they could be inactivated only by heating and prolonged incubation in the presence of non-specific proteases or certain denaturants. *E. tarda* cells have been observed to be surrounded by a layer of slime and this may help in bacterial adherence to host cells and also protect the bacteria from host defences. Besides, *E. tarda* is also able to penetrate or invade non-phagocytic cells such as HeLa, HEp-2 and epithelioma papillosum of carp, *Cyprinus carpio*, (EPC) cells (Janda et al, 1991;Ling et al, 2000; Marques et al, 1984).

I.1.4.2. Serum and phagocyte resistance

Serum and phagocyte-mediated killing are the two major defense mechanisms of non-specific immunity in fish (Dalmo et al, 1997). In order to survive and colonize in host cells, bacteria must overcome the primary immune response of the host system. Phagocytic attack is one of the first lines of defenses that the bacterial cells encounter after they have gained entry into the host. Opsonized virulent *E. tarda* strains were able to adhere to, survive, and replicate within fish phagocytes (Iida & Wakabayashi, 1993;Srinivasa Rao et al, 2001). They had the ability to circumvent the anti-bacterial defense by avoiding stimulation of reactive oxygen intermediates.

I.1.4.3 Toxins, enzymes and other secreted proteins

E. tarda could secrete various dermatotoxins and enzymes as virulence factors and these toxins induced erythema in mice. *E. tarda* also secrete two types of hemolysins, including cell associated and iron-regulated hemolysin, and extracellular hole-forming hemolysin (Chen et al, 1996; Hirono et al, 1997; Janda & Abbott, 1993). The cell-associated hemolysin was found to be an important virulence factor required for the invasive activities (Janda et al, 1991; Marques et al, 1984). A number of *E. tarda* strains produced chondroitinase which may aid in the destruction of host tissues and facilitate bacteria dissemination throughout the host body

system (Ruoff & Ferraro, 1987; Shain et al, 1996). Besides these, *E. tarda* also produce siderophores (Kokubo et al, 1990; Payne, 1988) and a 37 kDA toxin (Suprapto et al, 1996) that contribute to its pathogensis. *E. tarda* strains were also found to produce three different types of catalase-peroxidase (Kat1-3) of which KatB being the major catalase enzyme (Srinivasa Rao et al, 2003c). KatB was required for *E. tarda* survival and replication in phagocyte-rich organs in gourami fish, indicating its importance in virulence.

I.1.4.4. Type III secretion system in *E. tarda*

T3SSs are used by many bacterial pathogens for the delivery of virulence factors into the host cells. A comparison of the extracellular proteins (ECPs) of virulent and avirulent *E. tarda* strains revealed several major, virulent-strain-specific proteins. Proteomics analysis identified two of the proteins in the virulent strain. One is homologous to flagellin and the other protein spot (EseB) is homologous to the translocon protein SseB of *Salmonella* pathogencity island 2 (SPI-2) (Tan et al, 2002). The gene sequences were then identified using degenerate primers. At the same time, 490 alkaline phosphatase fusion mutants were screened from a library of 450,000 Tn*phoA* transconjugants derived from strain PPD130/91, using blue gourami fish as an infection host. Fourteen virulence genes were identified that were essential for disseminated infection, including enzymes, a phosphate transporter, novel protein and a protein similar to SsrB (EsrB), a regulator of *Salmonella* T3SS (Srinivasa Rao et al, 2003a). Based on the sequences of EseB and EsrB, the T3SS cluster was identified in the genome of *E. tarda*. The T3SS gene cluster from *E. tarda* PPD130/91 contained 35 open reading frames, and many of the putative genes were similar to those in SPI-2 T3SS of *S. enterica* serovar Typhimurium (Hensel, 2000; Hensel et al, 1998; Shea et al, 1996; Tan et al, 2005b). Thus, the designation of the *E. tarda* T3SS genes was based on the sequence homologs in *Salmonella* SPI-2. Similar to

Salmonella SPI-2, the genes of the *E. tarda* T3SS cluster were grouped into four categories: *E. tarda* secretion system chaperone (*esc*), effectors (*ese*) and regulators (*esr*) (Tan et al, 2005b). The mutation of the two-component system genes *esrA* and *esrB* led to near thousands folds decrease in LD_{50}. Furthermore, the adherence and invasion rates of *esrA* and *esrB* mutants were decreased while those of the apparatus and chaperone mutants remained similar to that of the wild type. It is proposed that the function of EsrB was possibly similar to the homologous global regulator SsrB in S*almonella*, which controlled several virulence factors encoded both inside and outside of the SPI-2 T3SS (Feng et al, 2003b;Gray et al, 2002). With 2D-PAGE analysis, EseB, EseC and EseD were identified as the major components in the ECPs and their secretions were T3SS-dependent. These three proteins contributed to the virulence of *E. tarda* as insertional mutations of them increased the LD_{50} values about 10 times (Tan et al, 2005b). Sequence analyses showed that these three proteins were homologous to EspA, EspD and EspB of EPEC, respectively. EspA formed a sheath-like structure, and EspB and EspD formed a translocon pore in enteropathogenic *E. coli* (EPEC). EspA, EspB and EspD together constituted a molecular syringe and channeled effector proteins into the host cell (Ide et al, 2001). In addition, the homologs of EseB, EseC, and EseD in *Salmonella* (SseB, SseC and SseD, respectively) were aslo shown to function as tranlocon components, and they were essential for the translocation of effectors (Nikolaus et al, 2001b). The bioinformatics results of EseBCD as translocon components were confirmed by the co-immunoprecipitation, which showed that EseBCD formed a complex after secretion (Zheng et al, 2007). A novel regulator, EsrC, which showed significant sequence similarity to the AraC family of transcriptional regulators is shown to regulate both T3SS and T6SS secretion system in *E. tarda*. Mutants with in-frame deletions of *esrC* have increased LD_{50} in blue gourami fish, reduced ECPs production

and failed to aggregate. Complementation of *esrC* restored the original wild type phenotypes. Analysis using 2D-PAGE showed that EsrC regulated the expression of secreted proteins encoded by the T3SS (such as EseB and EseD) and T6SS gene clusters. The expression of *esrC* is dependent on a functional two-component system of EsrA-EsrB. EsrC in turn regulates the expression of T6SS genes as well as selected T3SS genes (Zheng et al, 2005a). Temperature dependent expression of regulators was documented where the expression of the *esrB*-lacZ and *esrC*- LacZ fusions decreased substantially at 37°C compared to that seen at 25°C, but there was no change for the *esrA-lacZ* fusion at different temperatures. Although EsrA functions as a sensor in EsrA-EsrB two-component system, the expression of *esrB-lacZ* in an *ersA* mutant background did not decrease compared to that in the wild-type background at 25°C. This suggested the presence of other protein*s* which can respond to temperature changes (Zheng et al, 2005a).

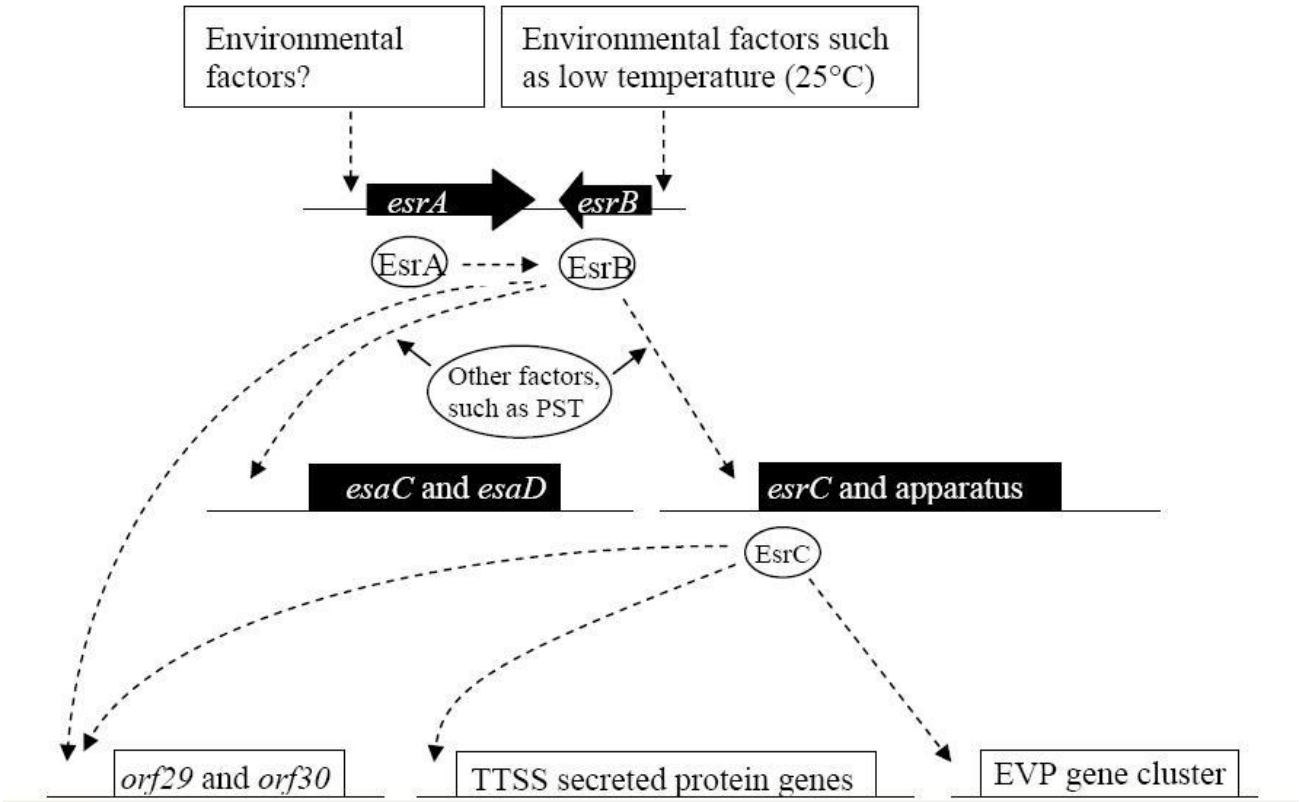

Fig. I.1. Model for the regulation of T3SS and EVP gene clusters by EsrA, EsrB, and EsrC in *E. tarda* PPD130/91.Growth in DMEM at 25°C favors the expression of *esrA* and *esrB*. EsrA is speculated to phosphorylate EsrB, and the accumulation of activated EsrB leads to the expressions of *esrC* and downstream T3SS apparatus genes, as well as *orf29* and *orf30*. EsrC then activates the expressions of T3SS secreted proteins, *orf29* and *orf30*, and the EVP gene cluster. The regulation of EsrB on the T3SS apparatus genes or *esrC* transcription was subjected to the modulation **(Adopted from Regulation of a type III and a putative secretion system in *Edwardsiella tarda* by EsrC is under the control of a two-component system, EsrA-EsrB. Zheng, J et al., 2005, with permission**).

I.1.4.5. Type VI secretion system in *E. tarda*

A comparison of secreted and total cell protein of the wild type *E. tarda* and those of Tn*phoA* highly attenuated mutants (*pstC*, *pstB*, *pstS*), identified three proteins absent from the Tn*phoA* highly attenuated mutants that did not belong to the T3SS (Rao et al, 2004). Based on the nano electrospray ionization (ESI) tandem MS data, EvpA and EvpC were identified. With genomics walking, an eight open-reading-frame gene cluster was sequenced and was named as the EVP gene cluster (Rao et al, 2004). Sequence blast search against NCBI GenBank revealed that this gene cluster was conserved in many other animal and plant pathogens and symbiont such as *Salmonella*, *Vibrio*, *Yersinia*, *Escherichia*, *Rhizobium* and *Agrobacterium* species. EVP proteins contribute to the pathogenesis of *E. tarda* as disruption of the EVP gene cluster resulted in about 2 logs increase of the LD_{50}s in the blue gourami host. The mutation of the EVP genes also led to lower replication rates in gourami phagocytes, and reduced protein secretion. In addition, *evpA* and *evpC* were shown to be regulated by the T3SS regulator *esrB* and the secretion of EvpC was dependent on EvpA. Mutations of the T3SS apparatus did not affect the secretion of EvpC, suggesting that the EVP gene cluster encodes a novel secretion system which is different from the T3SS in *E. tarda*. In the year 2007, entire T6SS gene cluster was sequenced using a combination of genome walking and phage library screening. The 16 genes present within the Type VI secretion system cluster were mutagenized to study the functions of this novel secretion system. All EVP mutants except Δ*evpD* were attenuated in blue gourami fish. The 16 EVP proteins were grouped based on cellular functions and localizations. The first group is the intracellular apparatus proteins which are non-secreted. Among them, EvpO, a putative ATPase which contained a Walker A motif, showed possible interactions with three EVP proteins (EvpA, EvpL and EvpN). Protein families with such

Walker A motifs in Gram-negative bacteria include ABC transporters (Nikaido, 2002) and T2SS proteins (Johnson et al, 2006). These proteins function as ATPases to energize secretion of their substrates. However, site-directed mutagenesis in the conserved amino acids of Walker A motif did not affect the secretion of EvpC, EvpI and EvpP, suggesting that the Walker A motif may not be essential for the secretion of these proteins. Thus, EvpO may have some functions other than ATPase. Yeast two-hybrid study demonstrated that three EVP proteins (EvpA, EvpL and EvpN) possibly interacted with EvpO suggesting interaction between T6SS proteins to form a secretion complex. The second group includes three secreted proteins (EvpC, EvpI and EvpP). EvpI, the homologue of VgrG (valin glycin repeat protein) in *E. tarda*, was not only a secreted protein but also a protein required for the secretion of other proteins, such as EvpC and EvpP. Similarly, the secreted protein EvpC (Hcp) was required for the secretion of EvpI (VgrG) and EvpP, which established its role as the secretion apparatus element of T6SS. This agrees with the fact that Hcp1, the homologue of EvpC in *P. aeruginosa*, forms a hexameric ring with a large internal diameter after secretion (Mougous et al, 2006). The secretion of EvpC and EvpI are mutually dependent, and they are required for the secretion of EvpP. Lastly, two proteins (EvpD and EvpJ) are not required for the T6SS-dependent secretion (Zheng & Leung, 2007b).

I.2. Secretion systems in gram-negative bacteria

I.2.1. Type I secretion system

The type I secretion system is indepandant of *sec* system and first described in the *E. coli* alpha-hemolysin (Salmond & Reeves, 1993). This secretion system requires three proteins: an inner-membrane ATPase (termed ABC protein for ATP-binding cassette), which provides energy for

the system (HlyB for *E. coli* hemolysin), a membrane fusion protein (HlyD) that spans the periplasm and the TolC out-membrane protein.

I.2.2. Type II secretion system

The type II secretion system is a *sec*-dependent protein secretion pathway which has been studied in many bacteria, such as *Klebsiella oxytoca*, *E. coli*, *Erwinia* spp., *V. cholerae*, *Pseudomonas aeruginosa* and *Aeromonas hydrophila* (Hacker & Kaper, 2000; Pugsley et al, 1997; Russel, 1998; Thanassi & Hultgren, 2000). Different from other secretion systems, proteins secreted into the extracellular milieu by the type II secretion system requires correct folding (Py et al, 1993). It is believed that recognition and outer membrane translocation of the secreted proteins occur once they have folded into a secretion-competent conformation (Filloux et al, 1998; Sandkvist, 2001).

I.2.3 Type III secretion system

The T3SS is a *sec*-independant secretion system generally composed of about 20 proteins, including apparatus, effectors, regulators, and chaperones (Hueck, 1998). The tranlocator proteins of the apparatus form a needle structure that is 28Å in diameter and deliver effector proteins into host cell membranes and cytosols (Marlovits et al, 2004). The recognition and secretion of effector molecules via the T3SS probably have different mechanisms. A 5' mRNA signal was required for the type III secretion of Yop proteins by *Yersinia enterocolitica P. syringae* and *Xanthomonas campestris* (Anderson & Schneewind, 1997; Anderson & Schneewind, 1999; Mudgett et al, 2000; Ramamurthi & Schneewind, 2003). However, the 5' mRNA signal was not found in other effector proteins (Karavolos et al, 2005; Lloyd et al, 2001).

I.2.4. Type IV secretion system

The type IV secretion system is also a *sec*-dependent protein secretion system through which the amino-terminal signal peptides of the secreted protein are removed (Hueck, 1998). Type IV secretion system could also mediate protein from a wide range of bacteria into host cells. Type IV secretion system has also been described in many other pathogenic bacteria, such as the *Bordetella pertussis* Ptl (pertussis toxin) system (Farizo et al, 2000), the cytotoxin-associated genes Cag Pathogenecity island (PAI) of *Helicobacter pylori* (Backert et al, 2002), and the Dot/Icm system of *Legionella pneumophila* (Vogel & Isberg, 1999). All these type IV secretion systems are required for DNA transfer and contribute to the virulence of pathogens

I.2.5. Type V secretion system

The type V secretion system is possibly the simplest *sec*-dependent protein secretion system. This secretion pathway encompasses the autotransporter proteins, the two-partner secretion system, and a type Vc or AT-2 family of proteins. The secreted protein and the secretion apparatus of type V secretion system are encoded in a single open reading frame rather than a cluster of genes encoding a multicomponent secretion apparatus in other secretion systems. (Henderson et al, 2004; Henderson et al, 1998).

I.2.6. Type VI secretion system

The type VI secretion system is found in several gram-negative bacteria, such as *Salmonella*, *Vibrio*, *Yersinia*, *Escherichia*, *Pseudomonas*, *Rhizobium*, *Agrobacterium, Burkhoholderia Aeromonas* and *Pseudomonas* (Bladergroen et al, 2003; Das & Chaudhuri, 2003; Folkesson et al, 2002; Ishikawa et al, 2009; Khajanchi et al, 2009; Moore et al, 2002; Mougous et al, 2006; Pukatzki et al, 2006; Schell et al, 2007; Suarez et al, 2008). Bioinformatics analysis showed that one protein encoded in each of these clusters had homology to the *Legionella pneumophila* IcmF

(Vogel & Isberg, 1999), and thus these clusters were designated as IcmF-associated homologous protein (IAHP) clusters (Das & Chaudhuri, 2003). Proteins encoded in the IAHP cluster of *R. leguminosarum* was reported to share limited homology with proteins encoded in the T3SS and T6SS, and the impairment of this cluster affected the secretion of at least one protein (Bladergroen et al, 2003). Folkesson and co-workers (2002) analyzed this cluster in *S. enterica* serovar Typhimurium and found that this gene cluster encoded putative cytoplasmic, periplasmic and outer membrane proteins. Pukatzki et al, (2006) found that the *vas* gene cluster, IAHP cluster homolog in *V. cholerae*, contributed to the pathogenesis in *Dictyostelium* as a model host. Their characterization studies showed that this cluster was involved in extracellular secretion of proteins lacking N-terminal hydrophobic leader sequences. Four proteins were found to be secretion dependent on the fully functional *vas* cluster. They proposed that this cluster was encoding a novel secretion system-type VI secretion system (Pukatzki et al, 2006). The role of HSI-I, one of three IHAP clusters in *P. aeruginosa*, was also investigated and was found to be essential for the secretion of Hcp1 (Mougous et al, 2006). ClpV, the ClpB homolog in this cluster, was found to form discrete foci in the bacterial cell. The mutation of *icmF* homolog and *hcp1* reduced the number of cells with these foci (Mougous et al, 2006). In addition, the ClpV proteins in EPEC and *S. enterica* servor typhimurium IAHP clusters formed hexameric structures that had ATPase activity, but lacked protein disaggregase activity (Schlieker et al, 2005). Thus, a role of ChpV as a core component of protein secretion apparatus to provide the energy for protein translocation was speculated. They further characterized the structure of the hexameric protein Hcp1 and found that it formed rings with a 40 Å internal diameter (Mougous et al, 2006). Thus, the IAHP cluster of *P. aeruginosa* was reported to encode the apparatus of this novel secretion system. Type VI secretion system also plays an important role in the virulence of

bacteria and it contributes to stress response, quorum sensing, biofilm production as well as survival within host phagocytic cells (Khajanchi et al, 2009; Lesic et al, 2009; Schell et al, 2007; Weber et al, 2009). Using silico analysis technique, Boyer and coworkers revealed 500 bacterial genomes with the presence of type VI cascades. Out of 500 bacteria, 92 of them were found to possess more than one copy of type VI clusters ranging from 2 to 6 (Boyer et al, 2009). Genes belonging to type VI clusters can be categorized into 3 major components depending on the functions and localizations. The 3 components are the secreted protein hemolysin co-regulated protein (Hcp), valin glycin repeat protein (vgrG) and the nonsecreted proteins (Boyer et al, 2009; Cascales, 2008; Pukatzki et al, 2006). Hcp proteins are responsible for the formation of extracellular tubule whereas, the VgrG is proposed to puncture the host membrane by means of a T4 phage like structure in order to deliver the effector proteins into the host cells (Ma et al, 2009; Pukatzki et al, 2007; Pukatzki et al, 2009).

I.2.7. Type VII secretion system

Recently, a novel type of protein secretion system has been identified in *Mycobacteria* which is also known as the ESAT-6 secretion pathway. The ESX-1 system is the archetype of type VII secretion system; this system is responsible for the secretion of 5 proteins, including the important T-cell antigens ESAT-6 and CFP-10 (Bitter et al, 2009). The substrates are synthesized without an amino-terminal Sec-type signal sequence, which suggests that the trans-envelope translocation machinery is independent of the Sec or Tat pathway. Periplasmic cargo proteins may be included in this process, but the specificity of recruitment remains elusive and the typing is therefore premature. Comparison of the secretion profile of wild-type strains and their ESX-5 mutants showed that a number of PE_PGRS and PPE proteins are dependent on ESX-5 for transport. Together, these data show that ESX-5 is a major secretion pathway for

mycobacteria and the ESX secretion systems form the paradigm for a new secretion pathway -- type VII secretion. This pathway is regarded as novel because: the T7SS is composed of a unique set of proteins; the main secreted proteins all belong to the same unique protein family (the ESAT-6/WXG100 family); and this pathway is mechanistically unlike any other secretion system that has been reported, as all the secreted proteins seem to be co-dependant on each other for secretion (Abdallah et al, 2007).

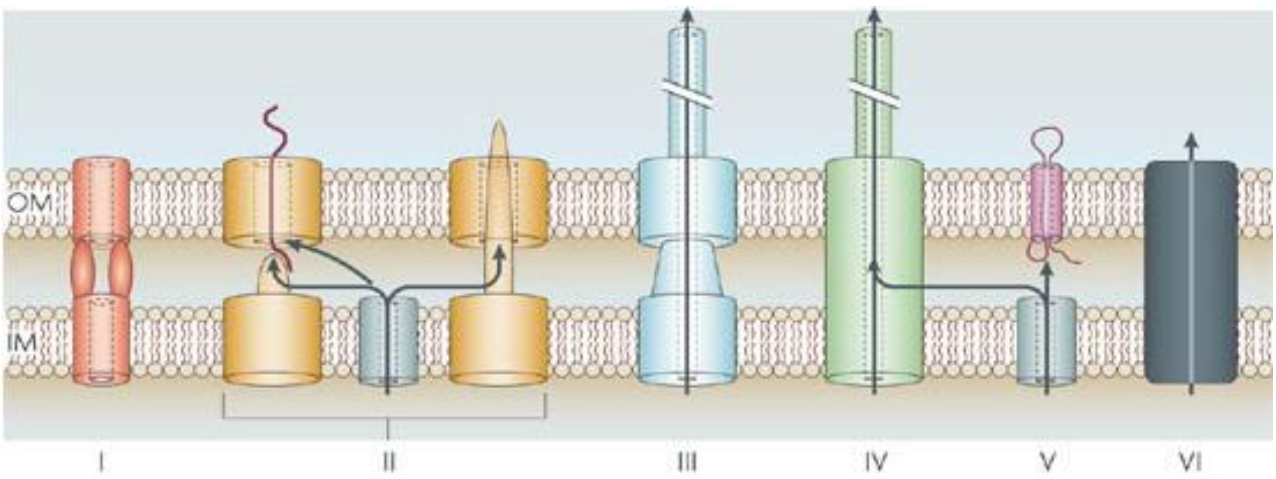

Fig. I.2. Schematic representation of Type I to Type VI secretion systems in Gram negative bacteria. Secretion in Gram-negative bacteria involves transport across a multipart cell envelope that consists of two membranes (the inner membrane (IM) and the outer membrane (OM)) and the periplasm in between. Gram-negative bacteria, and pathogenic species in particular, have developed strategies to get substrates into the extracellular milieu or directly into a host cell. Generally, secretion involves either a one-step mechanism, in which the cell envelope is crossed in one go, or a two-step mechanism, in which the OM is crossed using a specific machinery **(Adopted from Type VII secretion –Mycobacteria show the way, Abdallah M *et al*. *Nat Rev Microbiol* 5 (2007), 883-891.**

I.3 Cross-talk among Type III and Type VI regulatory systems

Regulation in different bacteria can be transcriptional, translational and post-translational events of which frequecny of transcriptional regulation is more predominant (Bernard et al, 2010). Multifaceted regulatory systems are required to impart spatial and temporal controls of type III gene expression (Francis et al, 2002;Hueck, 1998). Regulation is depandent on the enviornmetal stimuli which mimics conditions inside the host such as presense of cations, anti-microbial peptides, pH, temperature, osmolarity, quorum sensing and direct contact with host cells. Global regulators are mainly sigma factor proteins and two-component regulatory system. The predominant sigma factor proteins are alternate sigma factor (RpoS) and sigma factor 54 (RpoN) (Buchmeier et al, 1997; Doucleff et al, 2007; Francis et al, 2002; Potvin et al, 2008; Swords et al, 1997; Tu et al, 2006). In *Salmonella enterica*, RpoS stabilizes the two-component regulatory system PhoP/PhoQ (Tu et al, 2006). Most bacterial pathogens normally reside in the environments surrounding their hosts. Upon contact with the hosts, these bacteria sense the changes via a sensor-response regulatory system (a two-component system) to induce the expression of T3SS proteins. The sensor kinase is usually phosphorylated upon the signal stimulation, the phosphoryl group is then transferred to the response regulator, and thus increases the regulator's affinity for targeted DNA binding (Beier & Gross, 2006; Laub & Goulian, 2007; Rodrigue et al, 2000; Stock et al, 2000).

I.3.1 Cross-talk regulation in *Salmonella* sp and *E. tarda*

SPI-1 T3SS encodes many transcriptional regulators, including HilA, HilD, HilC (also called SprA/SirC), InvF and SprB among which HilA belongs to the OmpR/ToxR family, whereas HilD, HilC and InvF belong to the AraC/XylS family of transcriptional regulators (Kaniga et al, 1994; Lostroh et al, 2000; Lostroh & Lee, 2001).. In SPI-1, HilA play a key role in

coordinating expression of the SPI-1 T3SS. By binding upstream of the -35 sequences of P_{invF} and P_{prg}, HilA directly activates the expression of the *inv*/*spa* and *prg*/*org* operons that encode the components of the T3SS apparatus (Lostroh et al, 2000;Lostroh & Lee, 2001). The expression of HilA is subject to complicated regulation which involves regulators like RtsA, HilC and HilD. A mutation in HilD results in an approximately 10-fold decrease in *hilA* expression and 53-fold decrease in invasion of cultured epithelial cells (Schechter et al, 1999). However, a mutation in *hilC* results in a modest 50% decrease in *hilA* expression, and only a slight (20% to ~3-fold) decrease in invasion (Ellermeier & Slauch, 2003;Lostroh & Lee, 2001). Besides activating the expression of *hilA*, RtsA, HilC and HilD could also act independently of HilA to activate transcription of *invF* (Akbar et al, 2003; Ellermeier & Slauch, 2003). PhoP-PhoQ, which regulates virulent and physiological genes in a variety of gram-negative bacteria, represses expression of the SPI-1 T3SS in response to low magnesium concentrations and low pH (Behlau & Miller, 1993; Beier & Gross, 2006; Chamnongpol et al, 2003; Pegues et al, 1995; Prost & Miller, 2008b; Xu & Hensel, 2010). On the other hand, the PhoB-PhoR two-component regulatory system represses the expression of *hilA* in response to low extracellular Pi (Lucas & Lee, 2001; Lucas et al, 2000). Genes encoded in *Salmonella* SPI-2 T3SS are specifically induced inside both phagocytes and epithelial cells. Low concentration of Mg^{2+} or Ca^{2+}, as well as starvation of phosphate induce the expression of SPI-2 genes. These signals are probably perceived through the two-component systems, namely, EnvZ-OmpR and SsrA-SsrB. The two-component regulatory system SsrA-SsrB, encoded within SPI-2, controls the expression of the SPI-2 T3SS apparatus as well as the translocated effectors. The expression of SsrA-SsrB is in turn regulated by the two-component system OmpR-EnvZ, in which OmpR, binds directly to the *ssrA* promoter region and activates the transcription of *ssrA* (Feng et al,

2003b; Lee et al, 2000b; Xu & Hensel, 2010). SsrA-SsrB was found to be essential for the induction of SPI-2 gene expression in response to low osmolarity, acidic pH or the absence of Ca^{2+}, while OmpR-EnvZ seems to play a minor role in sensing these signals (Garmendia et al, 2003). The global regulator PhoP-PhoQ has also been reported to modulate SPI-2 gene expression (Deiwick et al, 1999; Hensel, 2000; Miller, 1991; Xu & Hensel, 2010). The regulation of PhoP-PhoQ on SPI-2 may be through modulation events upstream of OmpR as the PhoP-PhoQ regulatory system is not required for SPI-2 expression in the presence of OmpR~P (Kim & Falkow, 2004). The SPI-2 T3SS is found to be negatively regulated by the negative modulator YdgT. However, a mutation of *ydgT* also led to the attenuation of virulence (Coombes et al, 2005). *E. tarda* contains one copy of T3SS and T6SS each (Wang et al, 2009a) which are regulated by TRs similar to those found in *S. enterica,* PhoPQ and EsrAB in which EsrAB and EsrC are probably the primary crosstalk molecules. EsrB regulates most of the T3SS genes including *esrC,* which encodes a TR belonging to the AraC family (Zheng et al, 2005). Activated EsrC regulates T6SS genes and specific T3SS genes (Zheng et al, 2005). The ferric uptake regulator (Fur) extends the regulatory pathway to sense Fe^{2+} concentration. In the presence of iron, Fur binds to the promoter region of *evpP* and represses the transcription of T6SS genes presumably by blocking RNAP access (Wang et al, 2009b).

I.4 Objectives

The purpose of this PhD thesis, firstly, is to establish a regulatory cross-talk between the global regulators and the regulators present within the type III and type VI gene clusters. Previous reports from our group only identified the regulators which are present within the type III gene clusters. EsrC was under the control of EsrB and both of them were important for the secretion

of T3SS and T6SS proteins. However, cross-regulation between regulators outside the type III and type VI clusters and those within the clusters are unknown. Thus, deletion mutagenesis, gel retardation assay and reporter gene analysis were used to establish a cross regulation network which can supplement better understanding of the intricate regulation employed by bacteria to inject virulence proteins inside host cells. To understand the behaviour of pathogenic bacteria inside host cells, we identified six different environmental signals which mimic the conditions inside the host. The second objective is to extend the regulation study with the different environmental signals for a better understanding of the host-pathogen relationships. The third objective is to identify specific sensor proteins and to identify the key amino acid residues which are responsible for the recognition of specific environmental signals. We used approaches like circular dichorism, fluorescence, and site-directed mutagenesis to identify the key residues responsible for sensing the environmental signals. The forth objective is to identify the conditions which induce the up regulation and down regulation of regulatory proteins necessary for maintaining homeostasis in bacteria. The study aid in a better understanding of mechanism employed by pathogenic bacteria to inject virulence proteins inside host cells through the systematic functioning of sensor and regulator molecules in response to specific environmental signals. This study will also provide information on how regulatory proteins work together for the best interest of pathogenic bacteria inside host cells.

Chapter II. Common materials and methods

II.1. Bacterial strains, culture media and buffers

Complex medium such as TSA, TSB, LBA and LB were obtained from Becton-Dickinson Laboratories, USA and prepared according to the manufacturer's instructions. The media and broth cultures were autoclaved at 121°C for 20 min. For preparation of TSA or LBA along with TSB or LB, 1.5% Agar (Becton-Dickinson) was added and then sterilized at 121°C for 20 min. When the medium is cooled to 50°C, appropriate antibiotics were added if necessary, and the medium is poured into sterile petri-dishes (Sterilin, UK). To study the effect of magnesium, N-minimal medium (Nelson & Kennedy, 1971) was used at different incubation temperatures with 1 mM or 10 mM Mg^{2+}. For investigating the effect of phosphate and iron in *E. tarda* and its mutants, the bacterial cells were cultured in 5 ml Dulbecco's modified eagle medium (DMEM, Invitrogen, USA) without phenol red. KH_2PO_4 and $FeCl_2$ were added as an external source of in-organic phosphate and iron at the indicated conditions into DMEM to study the individualistic and additive effect of both phosphate and iron on the secretion and expression of specific proteins in *E. tarda*. Stock cultures of *E. tarda* and *E. coli* were maintained at -80°C as a suspension supplemented with TSB and LB broth containing 25% (v/v) glycerol, respectively When required, the culture media were supplemented with antibiotics (Sigma, USA) at the following concentrations unless otherwise stated: ampicillin (Amp, 100μg/ml), chloramphenicol (Cm, 30 μg/ml), colistin (Col, 12.5 μg/ml), kanamycin (Km, 100 μg/ml) and tetracycline (Tc, 12.5 μg/ml). Phosphate buffered saline (PBS) was used for washing and re-suspending the bacteria. PBS contains 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 and 1.4 mM KH_2PO_4 at pH 7.2.

II.2 Preparation of *E. tarda* cultures

A single colony of *E. tarda* strain was inoculated into 5 ml of culture media and incubated statically overnight at 30°C. A fresh culture was prepared the next day by transferring 0.5 ml of the overnight culture into a fresh tube with 5 ml TSB. The sub-cultured bacteria were then incubated for 2-3 h at 25°C until an optical density (OD) at 540 nm reached approximately 0.5 (OD_{550} 0.5 is predetermined as approximately 1×10^8 CFU/ml). The cells were harvested by centrifuging at 6,000 × g for 5 min at 4°C. The supernatant was discarded and the cells were washed twice with PBS. The bacterial cells were re-suspended in PBS and adjusted to OD_{540}=0.5 using a spectrophotometer (Shimadzu, UV1601, Japan).

II.3 Molecular biology techniques

II.3.1 Purification of DNA insert by PCR

The DNA insert was generated by PCR, using the genomic DNA purified from *E. tarda* as template. Upstream and downstream oligonucleotide primers containing restriction enzyme sites were used for PCR reaction. The 25 µl PCR reaction mix contains 0.5 µl of genomic DNA (0.1 µg/µl), 200 µM of each dATP, dCTP, dGTP and dTTP, 0.4 µl of each upstream and downstream primers, 1X Pfu buffer (20 mM Tris-Cl pH 8.8, 2mM $MgSO_4$, 10mM $(NH_4)_2SO_4$, 10mM KCl, 0.1% (v/v) Triton X-100, 0.1 mg/ml BSA) and 2.5 U of Pfu DNA Polymerase (Fermentas). PCR was carried out for 30 cycles at 95°C for denaturation, 52°C for annealing and 72°C for elongation. Both denaturation and annealing processes were performed with duration of 1 min and the elongation time was kept for 2 mins. The PCR product was analyzed on a 1% TAE agarose gel and purified using the Gel Extraction Kit (Qiagen) according to the standard manufacturer protocol. Other DNA polymerases used for PCR reactions are *Taq* DNA

polymerase (Invitrogen), Advantage® cDNA polymerase (Clontech) and Phusion® DNA polymerases (Finnzymes).

II.3.2 Purification of plasmid DNA

Plasmid DNA was purified using QIAGEN Spin columns (QIAGEN GmbH, Germany) or Wizard^{TM} Plus Minipreps DNA Purification System (Promega, USA). Bacterial strains containing plasmids were cultured in LB broth (with appropriate antibiotics) and incubated in an orbital shaker (Forma scientific, USA) with 225 rpm shaking at 37°C. After culturing for overnight, the plasmid DNA was extracted according to manufacturer's protocol and plasmid DNA was dissolved in 20 µl of elution buffer (10 mM Tris.Cl, pH 8.5). The quality and concentration of DNA were determined using a spectrophotometer (Shimadzu, UV-1601, Japan).

II.3.3 Genomic DNA isolation

For genomic DNA isolation, bacterial strains were grown in TSB culture at 25°C for overnight. Bacterial genomic DNA was extracted using QIAGEN Genomic DNA Purification Kit and the BIO 101 Genome DNA Kit according to the respective manufacturer's protocols. For the mini-preparation, the purified genomic DNA was dissolved in 50 µl of TE buffer (10 mM Tris-HCl, and 1 mM EDTA, pH 7.5) and stored in -20°C for future use.

II.3.4 Cloning and genome walking

II.3.4.1 Genome walking

Genome walking libraries were made according the manual of Clone Universal Genome Walker™ Kit. Briefly, 2.5 µg of purified genomic DNA was digested with different restriction enzymes including SmaI, ScaI, EcoRV, StuI and PvuII. The digested genomic DNA was further purified and ligated separately to the genome walker adaptor as follows:

4.0 µl purified restriction enzyme digested genomic DNA (approx. 0.5 µg)

1.9 µl of Genome Walker Adapter (25 µM)

1.6 µl 5× ligation buffer

0.5 µl T4 DNA ligase (1 unit/µl)

The ligation mix was incubated at 16°C for overnight followed by 5 min incubation at 70°C to stop the reaction.72 µl of TE (10 mM/1mM, pH 7.4) was then added to the ligation sample and mixed. The genome walking library was aliquoted (10 µl each) into several microcentrifuge tubes and stored at -20°C. PCR amplification was performed using primers that are specific to known upstream nucleotide sequence of mutants and the adaptor primer 1 (AP1). PCR was carried out using Advantage Polymerase 2 (Clontech, USA) and the cycling parameters were: 7 cycles each of 25 s at 94°C and 3 min at 72°C; 32 cycles each of 25 s at 94°C and 3 min at 65°C; and a final extension of 5 min at 65°C. After the PCR reaction 5 µl of PCR product was run on 1% agarose gel to analyze the result. A secondary PCR was carried out for those samples which gave multiple bands during the primary PCR reaction. The secondary PCR was done with the following two-step cycle parameters: 5 cycles of 25 sec at 94°C, 3 min at 72°C; 15 cycles of 25 sec at 94°C and 3 min at 67°C. The amplified fragments were cloned into

pGEM-T Easy vector system, transformed into *E. coli* JM109 competent cells and sequenced using AP1 and mutant specific primers.

II.3.4.2 Cloning and transformation into *E. coli* cells

PCR products were cloned into the pGEM-T Easy vector system (Promega, USA) according to the manufacturer's instructions and transformed into *E.coli* JM109. *E. coli* competent cells were prepared and transformed as according to the procedure provided by Sambrook and co-workers (1989). Transformants were plated on LBA containing Amp, IPTG, (Bio-Rad) and X-gal, (Bio-Rad), for blue-white colony selection.

II.3.5 Preparation of competent cells for heat shock and electroporation.

Glycerol stock of bacterial cells kept at -80°C was streaked and incubated at 37°C (for *E. coli*) and 30°C (for *E. tarda*) in a suitable agar medium without the addition of antibodies. Single fresh colony was inoculated in a fresh media at the required temperatures for overnight. 1 ml of the overnight culture was transferred into 50 ml of fresh medium at the required temperature in a shaker at a speed of 200rpm until O.D. reaches 0.5 at 600nm (for *E. coli*) and 550nm (for *E. tarda*). Cells were spun down at 6000 rpm at 4°C and the supernatant was discarded. To obtain competent cells for heat shock transformation, cell pellets were resuspended gently in 20 ml cold filter sterilized CCMB buffer (11.8 g $CaCl_2$,4.0 g $MnCl_2$,2.0 g $MgCl_2$,0.7 g KCl ,100 ml glycerol adjusted to pH to 6.4 with HCl, top up to 1 Liter with water). The cells pellets were washed twice and finally 2 ml of the same buffer was used to resuspend the cell pellets which were aliquoted (100µl per tube) and stored at -80°C until use. For competent cells meant for electroporation, ice cold 10% glycerol buffer was used as resuspension buffer.

II.3.6 Sub-cloning

The PCR product of the desired amplified gene was sub-cloned into pET-M, a modified pET-32a vector (Novagen, Darmstadt, Germany). 1 µg of purified PCR DNA product was digested by desired restriction enzymes in a total reaction volume of 20µl. Same combination of restriction enzymes was used to digest 1.5 µg of the pET-M vector in a separate tube. Both the insert and the vector were incubated at 37°C by using a water bath. Following the incubation, the digestion mixture of the DNA insert and the vector was purified by using QIA quick PCR purification kit according to the manufacturer's protocol. In the final step, 15µl of Elution buffer was used to elute out both the digested PCR product and the vector. The DNA insert and the plasmid were ligated in a 3:1 ratio in a single PCR tube by adding 1 µl of 10X T4 ligase buffer with 0.5 µl of T4 ligase enzyme (New England Biolab) topped up with sterile milli Q water to a final volume of 10 µl.

II.3.7. Transformation of ligation mixture into competent cells

II.3.3.7.1 Transformation using Electroporation:

Competent cells (100ul) from -80°C freezer were thawed on ice for 7 min. 10ul of ligation mixture was added into the 100ul competent cells and mixed by pipetting. The mixture was transferred to Gene Pulser Cuvette (Biorad) and put onto a holder. The pulse was applied to the mixture by using Biorad MicroPulser at a setting for bacteria, at "Ec 2". The cuvette was taken out from the holder after the 'Ti" sound. The mixture was transferred to a 1.7ml centrifuge tube and, 400µl of LB was added and the tubes were shaken at 37°C incubator for 2hrs and finally 100ul culture was plated onto plates containing suitable antibiotics for screening.

II.3.3.7.2 Transformation using Heat shock.

Ligation mixture was added into 100µl of competent cells and kept on ice for 30 min. Transformation was achieved by incubating the cells at 42°C for 90 seconds, followed by 2 minutes on ice. 400 µl of LB was added into the tubes and were shaken at 37°C incubator for 2hrs. Finally, 100ul culture was plated onto plates containing suitable antibiotics.

II.3.3.8. PCR screening of transformants

Insertion of DNA into the target plasmid was verified by colony PCR method. Selected colonies were half picked by using a sterile pipette tip and resuspended in 5 ul of sterile water. 2 ul of the suspension was used as a template for the PCR reaction. The correct colonies showing the correct PCR band size were cultured overnight to isolate the plasmid DNA. The plasmids DNA were verified by sequencing.

II.3.9 DNA Sequencing

DNA sequencing was carried out on an Applied Biosystems PRISM^{TM} 377 or automated DNA sequencer or ABI PRISM® 3100 genetic analyzer using the dye termination method. The ABI PRISM BigDye terminator Cycle Sequencing Ready Reaction Kit was used to perform sequencing PCR reaction (Applied Biosystems, USA). Sequencing PCR reaction was carried out in GeneAmp PCR system 2400 (Perkin Elmer, USA) with the following parameters: 96°C 10 sec, 50°C 5 sec, 60°C 4 min for 25 cycles. The final holding temperature was 4°C.

The PCR product was purified prior to automated sequencing. 40 µl of 75% (v/v) isopropanol was added to the sample and mixed. The tube was centrifuged at 13000× *g* for 20 min and the

pellet obtained was washed with 100 µl isopropanol by spinning at 13000× *g* for another 10min. Later, the pellet was air dried and stored at -20°C. Prior to sequencing, 4 µl of automated sequencing loading buffer [5× deionized formamide, 1× 25 mM EDTA, pH 8.0 with blue dextran (50 mg/ml)] was added to the pellet, heat denatured at 93°C for 2-3 min and loaded onto sequencing gel.

II.3.10 Sequence analysis

Sequence assembly and further editing were done using the DNA analysis softwar, DNASIS (Hitachi Software, Japan). BLASTX and BLASTP sequence homology and protein conserved domain analyses (CD-search) were performed by using the National Centre for Biotechnology Information BLAST network service (www.ncbi.nlm.nih.gov /BLAST; Altschul *et al*., 1990).

II.3.11. Isolation of RNA

mRNA was isolated from *E. tarda*, by using the RNAprotect™ Bacteria Reagent Handbook from QIAGEN according to manufacturer's protocol. *E. tarda* was grown overnight at 30°C in DMEM followed by sub-culturing into fresh DMEM until O.D reached a value of 0.5 at 550nm. The bacterial cell pellets were collected after centrifuging at a speed of 13,000 rpm for 10 minutes. Two volumes of the RNAprotect Bacteria Reagent were added and the reaction tubes were mixed immediately by vortexing for 5 seconds and then incubated for 5 min at room temperature. The pellets were collected after centrifuging at 55,000 rpm for 15 minutes. TE buffer containing lysozyme (1mg/ml) was added and the reaction mixture was vortexed for 10 seconds and then incubated at room temperature for 5 min. 700 µl of buffer RLT was added to the sample and the tubes were vortexed vigorously before the supernatant was collected for subsequent steps. 500 µl of 95% ethanol was added to the supernatant and mixed by pipetting. RNeasy Mini Protocol was used to obtain total RNA from *E. tarda*. The lysate containing

ethanol was applied to an RNeasy Mini Column placed in a 2 ml collection tube. The maximum loading volume was 700 µl. The tubes were centrifuged for 15 s at ≥8000 x *g* (≥10,000 rpm) and the flow through was discarded. 700 µl of Buffer RW1 was added to the RNeasy column and centrifuged for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the column. The flow through and the collection tubes were discarded. The RNeasy column was transferred into a new 2 ml collection tube and 500 µl Buffer RPE was added onto the RNeasy column followed by centrifugation for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the column. The flow through was again discarded. This step was repeated one more time followed by a short spin to remove any traces of Buffer RPE left inside the column. Finally to elute, RNeasy column was transferred to a new 1.5 ml collection tube and Pipet 30 µl RNase-free water was directly added onto the RNeasy silica-gel membrane; followed by centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm) for elution.

II.4 Protein techniques

II.4.1 Preparation of extracellular proteins from *E. tarda*

E. tarda strains were grown in DMEM at 25°C without shaking for 24 h [supplied with 5% (v/v) CO_2 atmosphere]. The bacteria were adjusted to an OD value of 0.8 at 550 nm and inoculated into fresh DMEM at a 1:200 dilution. The extracellular protein (ECP) fraction was isolated after 24 h by TCA–acetone precipitation (Shimizu et al., 2002). Briefly, the bacterial cultures were centrifuged at 4, 000 × g for 20 min at 4°C. The culture supernatant was then passed through a 0.22 µm, low-protein-binding Millex filter (Millipore, USA). The flow-through was precipitated with 10% (w/v) TCA for at least 1 h at 4°C. The protein pellet was washed with -20°C acetone thrice and then air dried. The protein pellet was dissolved in appropriated volume of ReadyPrep reagent 3 [5 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2%

(w/v) SB 3-10, 40 mM Tris, and 0.2% (w/v) Bio-Lyte 3/10 ampholyte (Bio-Rad, USA)]. Insoluble materials were removed by centrifugation at 14, 000 × g for 20 min at room temperature. Protein sample were stored at -86°C until analysis. The concentration of protein in the ECP samples was determined with the Bio-Rad Protein Assay. Briefly, 200 µl of dye reagent concentrate (Bio-Rad) were added to each tube (799 µl water + 1 µl protein) and the tubes were vortexed thoroughly and incubated at room temperature for 10 min. The mixtures were then subject to reading at OD_{595}. The protein concentration of each ECP sample was obtained based on the standard curve plotted using BSA protein of known concentrations.

II.4.2 One-dimensional polyacrlamide gel electophoresis (1D-PAGE)

1D-PAGE was performed according to a standard protocol (Sambrook et al., 1989) and 12% polyacrylamide gels were used for protein separation. Briefly, the resolving gel solution was poured into the gap between two glass plates and isopropanol was layered on top. The isopropanol overlay was poured off and the gel was washed several times with milli-Q water after the resolving gel was polymerized completely. The 5% stacking gel was poured on top of the resolving gel and a clean Teflon comb was immediately inserted. The Teflon comb was carefully removed after the stacking gel polymerized completely and the wells were washed with milli-Q water to remove any traces of unpolymerized acrylamide. Prior to loading, protein samples were mixed with the SDS gel-loading buffer [50 mM Tris-HCl, 100 mM DTT (Bio-Rad), 2% (w/v) SDS, 0.1% (w/v) bromophenol blue (Bio-Rad, USA), 10% glycerol] and boiled for 5 min. The samples were loaded into the gel well, and a constant current of 5 mA per gel was applied. After the dye front has moved into the resolving gel, the current was increased to 15 mA per gel. Tris-glycine electrophoresis buffer [25 mM Tris, 250 mM glycine (pH 8.3), 0.1% (w/v) SDS] was also used for the electrophoresis.

II.4.3 Two-dimensional polyacrlamide gel electophoresis

II.4.3.1 Iso-electric focusing (IEF)

Ettan™ IPGphor™ Isoelectric Focusing System (Amersham, USA) was used for the first-dimension isoelectric focusing according to the manufacturer's instructions. Briefly, the 18 cm Immobiline Drystrips with a linear or nonlinear gradient from pH range of 3 to 10 (Amersham, USA) were passively rehydrated for overnight (12 to 16 h) at room temperature with 340 µl rehydration buffer [8 M urea, 2% (w/v) CHAPS, 0.5% immobilized pH gradient (IPG) buffer, 50 mM dithiothreitol, and a trace amount of bromophenol blue]. 50 µl of rehydration buffer was added to the desired amount of protein sample (total volume < 30 µl) after rehydration, and cup loading was employed to load the protein sample just prior to IEF. IEF was performed using the following conditions: 150 V for 1:30 h, 500 V for 1.5 h, 4000 V for 1.5 h, 8000 V for 40000 Vh.

II.4.3.2 Second-dimensional PAGE

After IEF, the 18 cm IPG strips were equilibrated in equilibration buffer I (6 M urea, 2% (w/v) SDS, 50 mM Tris-HCl pH 8.8, 30% (v/v) glycerol and 130 mM DTT, trace amounts of bromophenol blue) for 10 min, followed by equilibration buffer II (6 M urea, 2% (w/v) SDS, 50 mM Tris-HCl (pH 8.8), 30% (v/v) glycerol and 135 mM iodoacetamide (Sigma, USA), trace amounts of bromophenol blue) for another 10 min. For second dimension SDS-PAGE, 12% polyacrylamide gels (20 x 20 cm, 1.0 mm thick) were used. The equilibrated-gel strips were placed on top of the 12% polyacrylamide gel. The gels were mounted in a PROTEAN II XL Cell (Bio-Rad) and the Tris-glycine electrophoresis buffer [25 mM Tris, 250 mM glycine

(pH 8.3), 0.1% (w/v) SDS] was used. The proteins were then resolved with a constant current of 25 mA per gel.

II.4.4 Silver staining of protein gels

Silver staining (Blum et al., 1987) was used for more sensitive detection of proteins in the gel. The gel was first fixed in 50% (v/v) methanol and 10% (v/v) acetic acid for at least 30 min, followed by 15 min in 50% (v/v) methanol. The gel was then washed 5 times (5 min each) with milli-Q water, followed by fresh 0.02% (w/v) sodium thiosulfate (Sigma, USA) for 1 to 2 min, and washed twice for 1 min each with milli-Q water. Freshly prepared, 0.2% (w/v) silver nitrate (Merck, Germany) solution was then added and the gel was stained for 25 min. The gel was then washed 1 min for twice with milli-Q water and developed (3% (w/v) sodium carbonate, 0.025% (v/v) formaldehyde (Sigma, USA)) until appropriate intensities of bands were achieved. The developing was stopped by adding 1.4% (w/v) EDTA for 10 min.

II.4.5 Western blot

Protein samples were subjected to 1D-PAGE and transferred onto Immun-Blot™ PVDF membrane [0.2 μm] (Bio-Rad, USA) with Semi-Dry transfer system (Bio-Rad, USA) using transfer buffer consisting of 100mM Tris (pH 7.4), 200 mM glycine and 20% (v/v) methanol. The membrane was then blocked using 5% (w/v) skim milk in phosphate buffered saline with 0.05% Tween 20 (Bio-Rad, USA) (TPBS) for overnight at room temperature. The next day, the membrane was incubated with primary antibody in 1% (w/v) skim milk in TPBS for 1 h and 20 min. The membrane was washed five times in TPBS and incubated with secondary antibody in TPBS for 1 h, followed by another five times wash in TPBS. Signal detection was performed using the SuperSignal WestPico Chemiluminescent substrate (Pierce, USA) and the Lumi-Film Chemiluminescent Detection film (Roche, USA).

II.4.6 Protein expression and purification

II.4.6.1 Protein expression

Plasmid DNA containing appropriate inserts was transformed into *E. coli* BL21 (DE3) cells to express the recombinant protein. A single colony of *E. coli* BL21 cells was cultured overnight in 50 ml LB containing 100 μg/ml Amp. 12 ml of overnight culture was inoculated into 1 liter of fresh LB broth containing 100 μg/ml Amp. Cultures were grown at 37°C in a shaker incubator until the O.D reaches 0.6 at 600nm. IPTG was aded at a final concentration of 0.5mM to induce the protein expression. After induction, cultures were either grown at 37°C for additional 8 hrs or at 20°C for overnight. Cells were harvested by centrifugation at 6,000 rpm for 20min at 4°C. Cells pellets were stored at -20°C for later use for purification.

II.4.6.2 Protein purification using Nickel-affinity chromatography

Cells from 1 liter culture were resuspended in 50 ml of Nickel binding buffer (20mM Tris-Cl pH7.9, 0.5 M NaCl and 5mM immidazole). The suspension was divided into 2 tubes and sonicated for 3 minutes with 35% amplitude. The sonication was carried out for 3 rounds and the lysate was collected by centrifuge at 10,000 rpm for 30 min at 4°C. Ni-NTA beads were charged with Charge buffer (50mM $NiSO_4$), washed three times with water and then equilibrated with Nickel binding buffer. The supernatant collected in the previous step was run through the column very slowly by using the peristaltic pump. The column was then washed three times with Nickel binding buffer and eight times with Nickel washing buffer (20mM Tris-Cl pH7.9, 0.5 M NaCl and 30mM immidazole). This helps to remove the unbound proteins from the column. His-tagged protein was eluted with 20 ml of Nickel elution buffer (20mM Tris-Cl pH7.9, 0.5 M NaCl and 500mM Immidazole). The eluted protein was dialyzed

overnight at 4°C at a suitable buffer before concentrating and running into a gel filtration column.

II.4.6.3 Gel filtration FPLC

Gel filtration FPLC (Fast protein liquid chromatography) was carried out in a Hiload 16/60 Superdex 75 pg (Amersham Biosciences) size exclusion chromatography column on AKTA FPLC system (Amersham Biosciences) using suitable buffers. Two column volumes of running buffer was used to pre-equilibrate and the flow rate was maintained at 1ml/min with a volume of 180ml. Eluted protein peaks were quantified by checking absorbance at 280nm and fractions were analyzed with SDS-PAGE.

II.4.7 Circular Dichorism spectropolarimetry

Circular dichroism (CD) measurements were performed using a J-810 spectropolarimeter (Jasco, Easton, MD) with a 1mm pathlength cuvette (Hellma, Müllheim, Germany). For thermal denaturation, 300µl of protein sample in suitable buffer was used. Thermal denaturation was monitored by changes in CD ellipticity at 218 nm or 206.5 nm as a function of temperature from 5°C to 80°C, with a heating rate of 2°C/min. Urea denaturation was performed by monitoring changes in the CD ellipticity at 210 nm. Protein samples with various amounts of urea were prepared in a buffer and were equilibrated for at least an hour at the required temperatures before measurement.

II.4.8 Fluorescence spectroscopy

Fluorescence spectra were performed using 2 µM of the protein sample in the presence of different concentrations of Mg^{2+} at 20°C, 30°C or 37°C. The sample was excited at 280 nm, and the emission spectra were recorded at 0.2nm intervals from 300 to 400 nm. Urea denaturation was monitored by changes in fluorescence at 350 nm. Samples containing 2 µM

of protein and different concentrations of urea were prepared in a suitable buffer presence or absence of 10 mM Mg^{2+}. The protein sample was equilibrated at 20°C, 30°C or 37°C for at least an hour before measurement.

II.5 Sequence alignment and secondary structure prediction

Amino acid sequence alignment was performed by using Clustal W, an online service hosted by the European Bioinformatics Institute Server (EBI: http://www.ebi.ac.uk/clustalw/). Amino acid sequences of all other bacteria homologs were obtained from GenBank (NCBI: http://www.ncbi.nlm.nih.gov/).Aligned amino acid sequences were compared based on their characteristic domains such as the transmembrane domain, amphipathic domain and conserved hydrophobic residues. The domain search was performed by using SMART (Simple Modular Architecture Research Tool) which gives us information regarding outlier homologs, PFAM domains, signal peptides, internal repeats and intrinsic protein disorder regions (Schultz *et al*. 1998) http://smart.embl-heidelberg.de/. The secondary structure prediction was performed using The PSIPRED Protein Structure Prediction Server from UCL Department of Computer Science (http://bioinf.cs.ucl.ac.uk/psipred/).

Table II.1 Bacterial strains and plasmid vectors used for this study

Strains or plasmids	Characteristics and Source*	Source/Reference
E. tarda		
PPD130/91	*Serpae tetra*, AVA, Singapore Neo^s, Col^r, Amp^s	(Ling et al, 2000)
E. coli		
DH 5α	*SupE44 Δlac U169 (ø80lacZΔM15)hsdR17 recA1 endA1 gyrA96 thi-1relA1;* Amp^s	(Hanahan, 1983)

BL-21 (DE-3)	F^- *omp*T *hsd*$S_B(r_B^-m_B^-)$ *gal dcm*	(Weiner, 1994)
JM109	Km^s, Col^s, Cm^s	Promega
MC1061(λpir)	*(λpir), thi thr-1 leu6 proA2 his-4 argE2lacY1 galK2 ara14 xyl5 supE44, λpir*	(Rubirés, 1997)
SM10 (λpir)	*thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu* Km^r *pir*	(Rubirés, 1997)
S17-1(λpir)	*thi pro hsdR hsdM*$^+$ *recA[RP42-Tc::Mu-*Km*::Tn7* (Tp^r Sm^r)Tra^+*]*	(Simon, 1983)
Plasmids		
pGEMT Easy	Amp^r	Promega
pRE112	Suicide vector; *R6K ori sacB* Cm^r	(Edwards *et al*, 1989)

II.6 Statistical analysis

All data were expressed as mean ± SD. The data were analysed using one-way ANOVA and a Duncan multiple range test (SAS software, SAS Institute, USA). Values of $P < 0.05$ were considered as significant.

Chapter III. Temperature sensing and regulation of virulence by a novel PhoP-PhoQ two-component system in *Edwardsiella tarda*

The results of this chapter are included in the following manuscript:

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Manuscript submitted

Abstract

The pathogenic bacterium *Edwardsiella tarda* employs both T3SS and T6SS to inject multiple effectors into eukaryotic cells.The PhoP-PhoQ two-component system is commonly used by bacteria to sense environmental factors. Here, we show that the PhoP-PhoQ system of *Edwardsiella tarda* detects changes in environmental temperature and Mg^{2+} concentration, as well as regulates the T3SS and T6SS preotins through direct activation of *esrB*. Protein secretion is activated from 23°C to 35°C or at low Mg^{2+} concentrations but it is suppressed at or below 20°C, at or above 37°C, or at high Mg^{2+} concentrations. The effects of temperature and Mg^{2+} concentration are additive. The PhoQ sensor domain has a low T_m of 37.9°C and it detects temperatures through a conformational change of its secondary structure. Mutation of specific Pro or Thr residues increased the stability of the PhoQ sensor drastically, altering its temperature sensing ability. The PhoQ sensor detects Mg^{2+} concentration through the direct binding of Mg^{2+} to a cluster of acidic residues (DDDSAD) and through changes that may affect its tertiary structure. Here, we describe for the first time the use of PhoP-PhoQ as a temperature sensor for bacterial virulence control.

Introduction

Edwardsiella tarda is a gram-negative bacteria pathogen which associates with septicemia and fatal infections in a wide variety of animals including fish and humans (Janda & Abott, 1993; Thune et al, 1993). Using functional genomics (Srinivasa Rao et al, 2003b) and proteomics (Srinivasa Rao et al, 2004) approaches, type III secretion system (T3SS) and type VI secretion system (T6SS, also named as EVP for *E. tarda* virulence proteins) have been identified as the two most important virulence mechanisms in *E. tarda* PPD130/91(Tan et al, 2005a; Zheng & Leung, 2007a). Interestingly, the expression and secretion of proteins from both T3SS, e.g. EseB, EseC and EseD, and T6SS, e.g. EvpA and EvpC, were found to be significantly suppressed at a growth temperature of 37^{o}C and virulence of *E. tarda* PPD130/91 grown at 37^{o}C was significantly reduced compared to that of the ones grown at 25^{o}C. In the same study, EsrB, a putative *E. tarda* secretion system regulator protein, was identified to affect expression of the above five proteins from T3SS and T6SS (Srinivasa Rao et al, 2004). Subsequent study has suggested the effect of temperature on the expression of *eseB*, *eseD*, and *evp* genes is through regulating the expression of *esrB*, but the actual mechanism and the proteins involved in sensing temperature changes were not determined (Zheng et al, 2005b).

Many bacteria employ two-component systems to sense environmental stimuli and to regulate differential gene expression for survival and virulence. The classical two-component system of bacteria consists of an inner membrane-bound sensor histidine protein kinase and a cognate cytoplasmic response regulator. The histidine kinase harbors a periplasmic N-terminal sensor domain that senses a specific stimulus. The information is transduced through intramolecular

conformational change at the transmembrane helices and HAMP linker domain (Aravind & Ponting, 1999) (named for its presence in histidine kinases, adenylyl cyclases, methyl-accepting chemotaxis proteins, and phosphatases) that resulted in activation of the cytoplasmic transmitter domain (dimerization domain and ATP binding domain) through autophosphorylation of a conserved His residue on the dimerization domain. The transmitter then activates its cognate receiver domain at the N-terminal region of the response regulator by transferring the phosphoryl group to a conserved Asp residue in the receiver domain. The response regulator gives rise to the appropriate cellular response, mediated by the C-terminal effector domain, mainly through protein-DNA interaction leading to differential gene expression. Dephosphorylation of the response regulator set the system back to the prestimulus state (Mascher et al, 2006).

The PhoP-PhoQ two-component system (PhoQ is the sensor histidine kinase and PhoP is the response regulator) is one of the most studied bacterial signaling system that senses environmental stimuli and regulates responses essential for survival and virulence of the bacteria. In *Salmonella*, the expression of two transporters that mediate Mg^{2+} uptake, the P-type ATPase MgtA and MgtB, is transcriptionally induced in low Mg^{2+} by the Mg^{2+} regulated PhoP-PhoQ two-component system. Mutants defective in *phoP* or in both *mgtA* and *mgtB* are hypersensitive to oxidative stress-dependent Fe (II)-mediated killing. The intracellular level of Mg^{2+} is tightly regulated by PhoP/PhoQ system for Mg^{2+} homeostasis and avoidance of metal toxicity (Chamnongpol & Groisman, 2002). Other than homeostasis, the PhoP-PhoQ two component system of *S. typhi* is also essential for virulence of the bacteria by allowing it to sense divalent cation, mildly acidic pH and antimicrobial peptide to provide a cue that the cell

is inside the phagosome of macrophage. The system then regulates many genes encoding virulence proteins with properties including intracellular survival, invasion, lipid A structure, resistance to antimicrobial peptides, and phagosome alteration (Prost & Miller, 2008a). At high Mg^{2+} concentration (mM level denotes an extracellular environment), the periplasmic domain of PhoQ adopts a conformation that inhibits its kinase activity and represses expression of PhoP activated genes. In a Mg^{2+}-deprived environment (μM level denotes an intracellular environment), these genes are activated and confers the bacteria the ability to survive inside macrophages (Groisman, 2001; Véscovi et al, 1996). The kinase activity of *S. typhi* PhoQ is also shown to be directly activated by antimicrobial peptides. The highly acidic surface of the PhoQ sensor domain has been proposed to participate in both divalent cation and antimicrobial peptide binding. Binding of antimicrobial peptide displace divalent cations between PhoQ metal binding sites and membrane phospholipid to initiate signal transduction (Bader et al, 2005). *S. typhi* also recognize acidic pH within the host macrophage phagosome through a structural mechanism by PhoQ. This conformational change is different from that in the presence of divalent cations or antimicrobial peptides and the activation effect is additive to that of antimicrobial peptide (Prost et al, 2007a; Prost et al, 2007b). Up to date, there is no report on the detection of temperature changes by the PhoP-PhoQ two-component system.

Growth temperature detection is essential for the survival and virulence of many pathogenic bacteria for recognition of the suitable host. For instance, the production of virulence protein EspB in enterohemorrhagic *Escherichia coli* (EHEC) 413/89-1 and enteropathogenic *E. coli* (EPEC) is temperature regulated and was found to be greatly increased when the strains were incubated at 37^{o}C compared to growth at lower temperatures (Ebel et al, 1996). The

expression of the virulence proteins EspA and EspB by EPEC are regulated by appropriate host body temperature in human (37^{o}C) and rabbit (39^{o}C). Secretion of these proteins by EPEC is maximal at 36^{o}C but decreased at 39^{o}C and stopped at 42^{o}C. In contrast, maximal secretion of these proteins by RDEC-1 (rabbit EPEC) occurred at 39^{o}C and continued at 42^{o}C (Abe et al, 1997). Virulence of *Shigella* spp. is also regulated by growth temperature. Strains phenotypically virulent when grown at 37^{o}C are phenotypically avirulent when grown at 30^{o}C. Strains grown at 33^{o}C were only partially invasive, whereas strains grown at 35^{o}C were as invasive as those grown at 37^{o}C (Maurelli et al, 1984). The temperature detection mechanism has been determined in some of the bacteria. In *Listeria monocytogenes*, expression of the virulence genes (maximally expressed at 37^{o}C and silent at 30^{o}C) is regulated by a transcriptional activator, PrfA. The untranslated mRNA (UTR) preceding *prfA* forms a secondary structure that mask the ribosome binding region and this UTR is shown to switch between an active structure at high temperatures and an inactive one at low temperatures (Johansson et al, 2002). The two-component system has also been reported previously for detection of temperature. The DesK-DesR two component system of *Bacillus subtilis* activates transcription of the *des* gene, coding for an acyl-lipid desaturase, at low growth temperature to increase synthesis of unsaturated fatty acid to improve membrane fluidity (Cybulski et al, 2002). The membrane domain of DesK is shown to be the temperature-sensing element of the two-component system as the kinase function of the cytosolic domain of DesK alone is temperature independent and caused a constitutive expression of the *des* gene (Hunger et al, 2004). However, the exact temperature sensing mechanism by the membrane domain of DesK is unknown.

Here, we report identification of a novel PhoP-PhoQ two-component system in *E. tarda* that is involved in sensing growth temperature and Mg^{2+} for regulation of expression of virulence proteins from both T3SS and T6SS through PhoP activated transcription of EsrB. The periplasmic sensor domain of PhoQ is responsible for sensing temperature by a conformational change that is highly sensitive to temperature. Using thermal and urea denaturations combined with site-directed mutagenesis, we have identified residues in the PhoQ sensor domain that are essential for temperature sensing. Our findings provide an understanding of the mechanism of temperature detection by protein sensor and also shed light on the temperature and Mg^{2+} regulation of virulence in pathogenic bacteria.

III.2 Materials and Methods

III.2.1 Cloning of the PhoP-PhoQ two-component system in *E. tarda* PPD130/91

Bacterial genomic DNA was extracted using the Wizard genomic DNA purification kit (Promega, Madison, WI). PCR amplification (2 min at 94°C; 30 cycles each of 10 s at 94°C, 30 s at 56°C and 1 min at 72°C; and a final extension of 5 min at 72°C) was carried out using the Advantage 2 polymerase mix (Clontech, Mountain View, CA) with two pairs of degenerate primers, *phoP*deg and *phoQ*deg. The PCR products were cloned with the pGEM-T Easy vector system (Promega) and transformed into *E. coli* DH5α cells. The cloned fragments were sequenced using the PRISM^{TM} 3100 automated DNA sequencer with the ABI Prism Big Dye termination cycle sequence kit (Applied Biosystems, Foster City, CA). This approach identified a 400-bp fragment of the *phoP* gene, which was later used to design primers for genome walking. To obtain the full length sequences of *phoP* and *phoQ*, genome walking libraries of *E. tarda* PPD130/91 were created and digested with SmaI, ScaI, EcoRV,

Table III.1. Primers used in this study

Name	Sequence (5' to 3')
*phoP*deg-for:	T A/T CTGGTTGT A/T/G GAGGA T/C AA T/C GCA
*phoP*deg-rev	AA A/C AGATA T/G CC T/C TG T/G CC G/A GC
*phoQ*deg-for	CCAT C/T GTG GTGGT C/G GAT A/T CCATTC
*phoQ*deg-rev	TGCAGGTA A/G GTAGCC G/A GATTCTGCTG
pRW*esrb*-for	ATGAATTCGCCCATCGATACTGAGCGTGA
pRW*esrb*-rev	ATAAGCTTTACGCTAAAAGGGCTGGCCG
pRW*phoP*-for	ATGAATTCCGAGGAGTAAATGGCGGGGC
pRW*phoP*-rev	ATAAGCTTCGACGATCGCGATATCAGGG
$phoQ_s$-for	ATGGATCCTTCGACAAGACCACCTATCG
$phoQ_s$-rev	ATGAATTCTCACTCTTGGGGGATGGTATCGAC
$phoQ_f$-for	ATGGATCCATGATGCTACAGCGCTGGC
$phoQ_f$-rev	ATGAATTCTTAATCACAATCCGGCTGCTG
$phoP_{mut}$-for	GGGGTACCCCAACAGAGCCCTGATATCG
$phoP_{mut}$-rev	GGGGTACCCCGTCTCTATGATGGTGTAC
$phoQ_{mut}$-for	GGGGTACCCCCGTATCTCCCAGCAGATC
$phoQ_{mut}$-rev	GGGGTACCCCGCTGACCTCCACAAACTC
$phoP_{full}$-for	ATAGTACTATGCGTATCTTAGTCGTCGA
$phoP_{full}$-rev	ATGAATTCTGCCGGCACGTCGAAGCGGTAG
$phoQ_{full}$-for	ATAGTACTATGATGCTACAGCGCTGGCGGGC
$phoQ_{full}$-rev	ATGAATTCATCACAATCCGGCTGCTGCT
$phoQ_{EPEC\_full}$-rev	CGGAATTCTTATTCATCTTTCGGCGCAGA

StuI and PvuII according to the procedure described in the Universal Genome Walker kit manual (Clontech). PCR amplification (7 cycles each of 25 s at 94°C and 3 min at 72°C; 32 cycles each of 25 s at 94°C and 3 min at 65°C; and a final extension of 5 min at 65°C) was carried out using primers specific for sequences of *phoP* and *phoQ*, with the adaptor primer 1 (Clontech). The complete sequences of *phoP* and *phoQ*, including flanking sequences comprising the 1,260 bps upstream of *phoP* and 1,296 bps downstream of *phoQ*, were obtained by this method.

Table III.2. Strains and plasmids for this study

Strain/plasmid	Description	Reference/Source
E. tarda		
E. tarda PPD130/91	Wild type, Km^s, Col^r, Amp^s, $LD_{50}=10^{5.0}$	(Ling et al, 2000)
$phoP_i$	*phoP*::pRE112-*phoP* Cm^r	This study
$phoQ_i$	*phoQ*::pRE112-*phoQ* Cm^r	This study
$phoP_i$ + *phoP*	$phoP_i$ with pACYC + *phoP*	This study
$phoQ_i$ + *phoQ*	$phoQ_i$ with pACYC + *phoQ*	This study
$phoQ_i$ + *phoQ*(P140H)	$phoQ_i$ with pACYC + *phoQ*(P140H)	This study
$phoQ_i$ + *phoQ*(T76E)	$phoQ_i$ with pACYC + *phoQ*(T76E)	This study
$phoQ_i$ + *phoQ*(P77L)	$phoQ_i$ with pACYC + *phoQ*(P77L)	This study
$phoQ_i$ + *phoQ*(T167P)	$phoQ_i$ with pACYC + *phoQ*(T167P)	This study
$phoQ_i$ + *phoQ*(P79E)	$phoQ_i$ with pACYC + *phoQ*(P79E)	This study
$phoQ_i$ + *phoQ*(P120N)	$phoQ_i$ with pACYC + *phoQ*(P120N)	This study
$phoQ_i$ + $phoQ_{EPEC}$	$phoQ_i$ with pACYC + *phoQ*(EPEC)	This study
$pRWphoP_{130/91}$	pRW50 containing -1460 to +150 of *phoP* relative to putative translational start site in *E.tarda* PPD130/91	This study

pRWesrB$_{130/91}$	pRW50 containing -900 to +150 of *esrB* relative to putative translational start site in *E.tarda* PPD130/91	This study
pRWesrB$_{phoPi}$	pRW50 containing -1460 to +150 of *esrB* relative to putative translational start site in *E.tarda phoP$_i$* mutant.	This study
pRWesrB$_{phoPi + phoP}$	pRW50 containing -1460 to +150 of *esrB* relative to putative translational start site in *E.tarda phoP$_i$*+*phoP* complemented strain	This study
E. coli		
EPEC 2348/69	Prototypic EPEC strain, O127:H6, Sm^r	(Tu et al, 2003)
pETM-phoQ$_s$	PhoQ sensor domain in Bl-21 cells cloned in pETM	This study
pETM-phoQ$_f$	Full length PhoQ in Bl-21 cells cloned in pETM	This study
pETM-phoP	PhoP in Bl-21 cells cloned in pETM	This study
Plasmid		
pGEM-T Easy	Amp^r	Promega
pRE112	pGP704 suicide plasmid, pir dependent, Cm^r, oriT, oriV, sacB	(Edwards, 1998)
pACYC184	Tet^r, Cm^r	Amersham
pRW50	lacZ reporter vector, Tet^r	(Lodge, 1992)
pETM	modified pET-32a plasmid	Novagen

III.2.2 LacZ reporter gene system

For the construction of the *ersB* LacZ reporter gene system, the primer pair, pRW*esrb*, derived from *E. tarda* PPD130/91 genomic DNA, was used to amplify the putative promoter region of *esrB* containing a PhoP box. The PCR product was digested with restriction enzymes and then ligated with the pRW50 plasmid (Lodge et al, 1992). The ligation mixture was introduced into either the wild type or mutant *E. tarda* by electroporation. The transformants were screened for resistance to both colistin (12.5μg/ml) and tetracycline (10μg/ml). Similar procedures were followed to generate the *phoP* and *phoQ* LacZ reporter gene systems using the primers pRW*phoP* and pRW*phoQ*, respectively.

For the β-galactosidase assays, *E. tarda* cells were grown in N-minimal medium (Nelson & Kennedy, 1971) overnight at 20°C, 23°C, 25°C, 30°C, 35°C or 37°C with 1 mM or 10 mM Mg^{2+}. The overnight culture (5%) was inoculated into fresh medium and grown at the specified temperatures until the cell density reached 0.5, as measured by optical density at 600 nm (OD_{600}). Cells were permeabilized (Miller, 1972) by the addition of 25 μl of 0.1% SDS and 25 μl of chloroform to a solution containing the cell pellet from a 1-ml culture resuspended with 600 μl of Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO4, 10 mM KCl and 1 mM $MgSO_4$, pH 7.0). To start the reaction, 100 μl of ONPG (4 mg/ml) was added. When the suspension started to turn yellow, the reaction was terminated by adding 250 μl of 1 M Na_2CO_3. The mixture was centrifuged for 5 min at 13,000 × *g*, and the supernatant was transferred to a cuvette to measure the absorbance at 420 nm. The cell density was assessed by the OD_{600}, and the unit of activity was calculated as follows: (absorbance at 420 nm) / (reaction time in min × OD_{600}).

III.2.3 Electrophoretic mobility shift assay

The full length PhoP protein at various concentrations (0, 0.5, 1, 1.5 or 2 μM) was mixed with purified *esrB* or *phoP* promoter DNA fragments (2 μg) labeled 5'with 6-carboxyfluorescein (6-FAM) tag (1st Base, Singapore) in a reaction mixture (50 μl) containing 50 mM Tris-HCl, pH 7.5, 200 mM KCl, 0.1 mM EDTA and 5% v/v glycerol. The mixture was incubated at 25^{o}C for 2 h before loading (15 μl) onto each lane of a 5% native polyacrylamide gel for electrophoresis (0.5X Tris borate-EDTA buffer, 1 mA/cm for 4 h).

III.2.4 Western blot analysis

Proteins were separated on 12 % SDS-polyacrylamide gels. For western analyses, proteins were transferred to a PVDF membrane with a semi-dry system and examined by using the SuperSignal WestPico Chemiluminescent substrate (Pierce, Rockford, IL) under the conditions recommended by the manufacturer. EseB and EseC were detected by the addition of diluted anti-EseB (1:10000) and anti-EseC (1: 10000) polyclonal antisera, respectively, followed by a 1:5000 dilution of mouse anti-rabbit IgG HRP (Santa Cruz Biotechnology, Santa Cruz, CA).

III.2.5 Cloning, expression and purification of the PhoQ sensor

The PhoQ periplasmic sensor domain (from residue Phe47 to Glu186) was amplified by using the primer pair, *phoQ*$_s$, derived from *E. tarda* PPD130/91 genomic DNA. The PCR product was sub-cloned into pET-M, a modified pET-32a vector (Novagen, Darmstadt, Germany) (pETM-*phoQ*$_s$), and then transformed into *E. coli* BL21(DE3) cells for expression. To generate pETM-*phoQ*$_f$, the full-length phoQ gene was amplified using the primer pair, *phoQ*$_f$ and sub-cloned into pET-M. An overnight culture of pETM-phoQ$_s$ in BL21(DE3) cells was inoculated into LB with 100 μg/ml of ampicillin, and the cells were grown until the cell density

reached 0.8 at OD_{600}. Protein expression was induced with 0.5 mM of IPTG, and the cells were grown at 30^{o}C overnight. Cells were harvested by centrifugation and resuspended in 50 mM sodium phosphate, pH 8.0 and 300 mM NaCl. The cell suspension was sonicated and then centrifuged to obtain the inclusion bodies as a pellet. The inclusion bodies were resuspended in 20 mM sodium phosphate, pH 8.0, 100 mM NaCl and 8 M urea and lysed by sonication. The lysate was centrifuged and the supernatant was purified using a Ni-NTA affinity column in buffer containing 8M urea. Refolding was performed by rapidly diluting the eluate into an ice-cold solution of 20 mM sodium phosphate, pH 8.0 containing 0.1 or 10 mM Mg^{2+}, with stirring. The refolded sample was dialyzed overnight against a buffer containing 20 mM sodium phosphate, pH 6.5 and 100 mM NaCl and in the presence or absence of 10 mM Mg^{2+}. The sample was further purified by a Superdex-75 gel filtration column (GE Healthcare, Piscataway, NJ) equilibrated with the dialysis buffer. Fractions containing PhoQ sensor were pooled and concentrated. A similar procedure was followed for the purification of the various PhoQ mutants.

III.2.6 CD monitoring of the thermal and urea denaturation of PhoQ sensor

Circular dichroism (CD) measurements were performed using a J-810 spectropolarimeter (Jasco, Easton, MD) with a 1-mm pathlength cuvette (Hellma, Müllheim, Germany). For thermal denaturation, a 300-µl sample of 15 µM PhoQ sensor in 20 mM sodium phosphate, pH 6.5 and 100 mM NaCl and in the presence or absence of 10 mM Mg^{2+} was used. Thermal denaturation was monitored by changes in CD ellipticity at 218 nm or 206.5 nm as a function of temperature from 5^{o}C to 80^{o}C, with a heating rate of 2^{o}C/min. Urea denaturation was performed by monitoring changes in the CD ellipticity at 210 nm. Samples containing 20 µM

of the PhoQ sensor and various amounts of urea were prepared in a buffer containing 20 mM sodium phosphate, pH 6.5 and 100 mM NaCl in the presence or absence of 10 mM Mg^{2+}. The protein sample was equilibrated for at least an hour at 20°C, 30°C or 37°C before measurement.

III.2.7 Fluorescence spectra and urea denaturation of the PhoQ sensor

Fluorescence spectra were performed using 2 μM of the purified PhoQ in 20 mM sodium phosphate, pH 6.5 and 100 mM NaCl in the presence of different concentrations of Mg^{2+} at 20°C, 30°C or 37°C. The sample was excited at 280 nm, and the emission spectra were recorded at 0.2-nm intervals from 300 to 400 nm. Urea denaturation was monitored by changes in fluorescence at 350 nm. Samples containing 2 μM of the PhoQ sensor and different concentrations of urea were prepared in a buffer containing 20 mM sodium phosphate, pH 6.5 and 100 mM NaCl in the presence or absence of 10 mM Mg^{2+}. The protein sample was equilibrated at 20°C, 30°C or 37°C for at least an hour before measurement.

III.2.8 Generation of *phoP*$_i$ and *phoQ*$_i$ mutants and complementation experiments

Insertional mutants of *phoP* (*phoP*$_i$) and *phoQ* (*phoQ*$_i$) in *E. tarda* were constructed with the suicide plasmid pRE112 (Edwards et al, 1998). For the construction of *phoP*$_i$, an internal fragment of *phoP* was amplified from *E. tarda* genomic DNA with the primer pair, *phoP*$_{mut}$, which contains a *Kpn* I restriction enzyme site. The PCR product was digested by restriction enzymes and ligated into the pRE112 plasmid, and the resulting plasmid was transformed into *E. coli* MC1061 λpir. After sequencing, the recombinant plasmid was then transformed into *E. coli* SM10 λpir. These transformants were used to conjugate with wild type *E. tarda* to obtain defined mutants by selecting colonies resistant to both chloramphenicol (30 μg/ml) and colistin

(12.5 μg/ml). The insertion of the plasmid into chromosomal DNA was confirmed by sequence analysis. The primer pair $phoQ_{mut}$, was used for the construction of $phoQ_i$.

Complementation of the $phoP_i$ and $phoQ_i$ mutants was performed with a pACYC184-based system. To obtain $phoP_i$ + *phoP*, the complete *phoP* gene was prepared by PCR using the primer pair, $phoP_{full}$ from *E. tarda* PPD130/91 genomic DNA. The PCR product was digested with restriction enzymes and ligated into the digested pACYC184 plasmid. The obtained ligation mixture was transformed into *E. coli* DH5α, and then the plasmid DNA was extracted and transformed into competent cells of *E. tarda* $phoP_i$ by electroporation. The same procedure was followed to obtain $phoQ_i$ + *phoQ* using the primer pair, $phoQ_{full}$.

III.2.9 Gram staining and Microscopic analysis.

E. tarda bacterial cells grown overnight in TS at 20°C, 35°C and 37 °C followed by subculturing until the $O.D_{550nm}$ reaches 0.5. Cells were harvested and smeared on glass slides. Gram staining was performed according to manufacturer's protocol (*Clinical Microbiology Procedures Handbook, 1992*). The bacterial cells were gram stained and the microscopic images were obtained using Nikon Eclipse 90i objective: Plan Apo VC 60x/1.40 Oil at scale bar of 2 μM.

III.3 Results

III.3.1 Identification of the PhoP-PhoQ two-component system

Using the genome walking approach with degenerate primers derived from conserved nucleotide sequences of the *phoP* and *phoQ* genes of related bacterial species, we have identified the presence of these genes in the genome of *E. tarda* PPD130/91. Protein domain searches using SMART (Letunic et al, 2009; Schultz et al, 1998) showed that PhoQ is a sensor

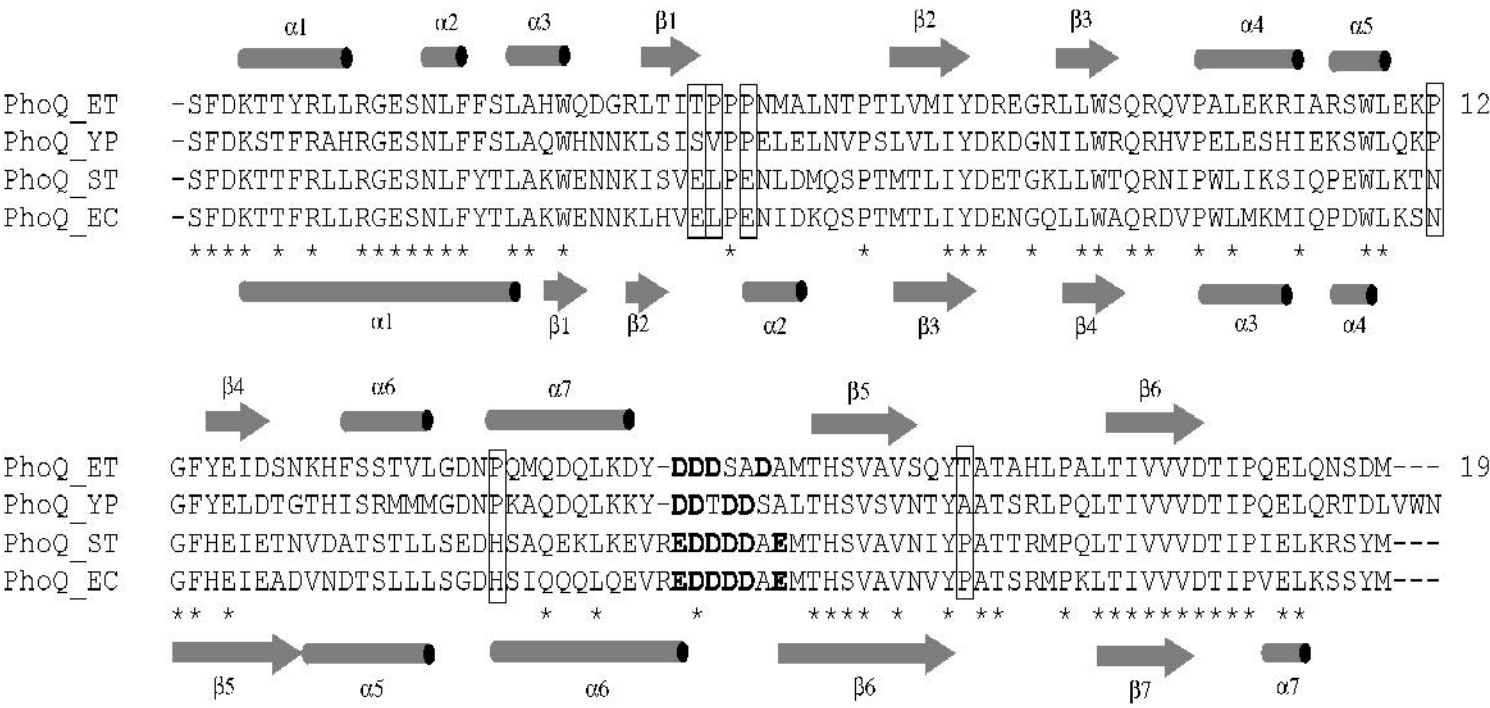

Fig. III.1. Sequence alignment of the PhoQ sensor domains. The sequence of the PhoQ periplasmic sensor region from *E. tarda* (PhoQ_ET; GU324976) was compared with those from *Y. pestis* (PhoQ_YP; *NP_669110*), *S. typhimurium* (PhoQ_ST; *NP_460200*) and *E. coli* (PhoQ_EC; *NP_753417*). The secondary structural elements from the crystal structure of the *E. coli* PhoQ sensor are shown underneath the sequences, while the predicted secondary structural elements of the *E. tarda* PhoQ sensor are shown on top of the sequences. Asterisks below the sequences indicate identical residues. Negatively charged residues in the acidic patches are bold-faced. Pro and Thr residues in the *E. tarda* PhoQ sensor that are not conserved with those in *E. coli* and *S. typhimurium* and were selected for mutation studies are boxed.

histidine kinase consisting of a N-terminal periplasmic sensor domain spanned between two transmembrane α-helices; a cytosolic HAMP linker; and a C-terminal histidine kinase domain containing both the dimerization and ATPase domain. The PhoQ sensor domain is rich in acidic residues and likely to be involved in cation binding. The PhoP protein is a response regulator consisting of a N-terminal CheY-like receiver domain with a phosphoacceptor site and a C-terminal DNA binding domain, likely for transcription regulation. Sequence alignment of the periplasmic PhoQ sensor domain of *E. tarda* shows 50.0% identity to *Salmonella enterica serovar Typhimurium LT2* (McClelland et al, 2001a), 59.7% identity to *Yersinia pestis KIM* (Deng et al, 2002b), and 50.0% identity to *Escherichia coli CFT073* (Welch et al, 2002b). However, the sequence identity between the PhoQ sensor domain of *E. coli* and *S. typhimurium* is very high and determined to be 81.1% (Fig. III.1). The relatively low sequence identity of the PhoQ sensor domain of *E. tarda* as compared to those of *E. coli* and *S. typhimurium* suggests that it may sense a very different extracellular stimulus. The secondary structure of the PhoQ sensor domain of *E. tarda* using PsiPred (Bryson et al, 2005; Jones, 1999) is shown in Fig. III.1. The crystal structures of the PhoQ sensor domain of *S. typhimurium* (Cho et al, 2006) and *E. coli* (Cheung et al, 2008) are available. Both structures adopt a mixed α/β-fold containing a central five-stranded anti-parallel β-sheet flanked by a long N-terminal α-helix and additional loops and helices on each side. This fold is characteristic of the PAS (Per-Arnt-Sim) domain superfamily (Taylor & Zhulin, 1999) often found in other bacterial histidine kinase family such as DcuS (Pappalardo et al, 2003) and CitA (Reinelt et al, 2003), which are bacterial receptors for fumerate and citrate, respectively. The predicted secondary structures of *E. tarda* PhoQ sensor domain agree with those from the 3D

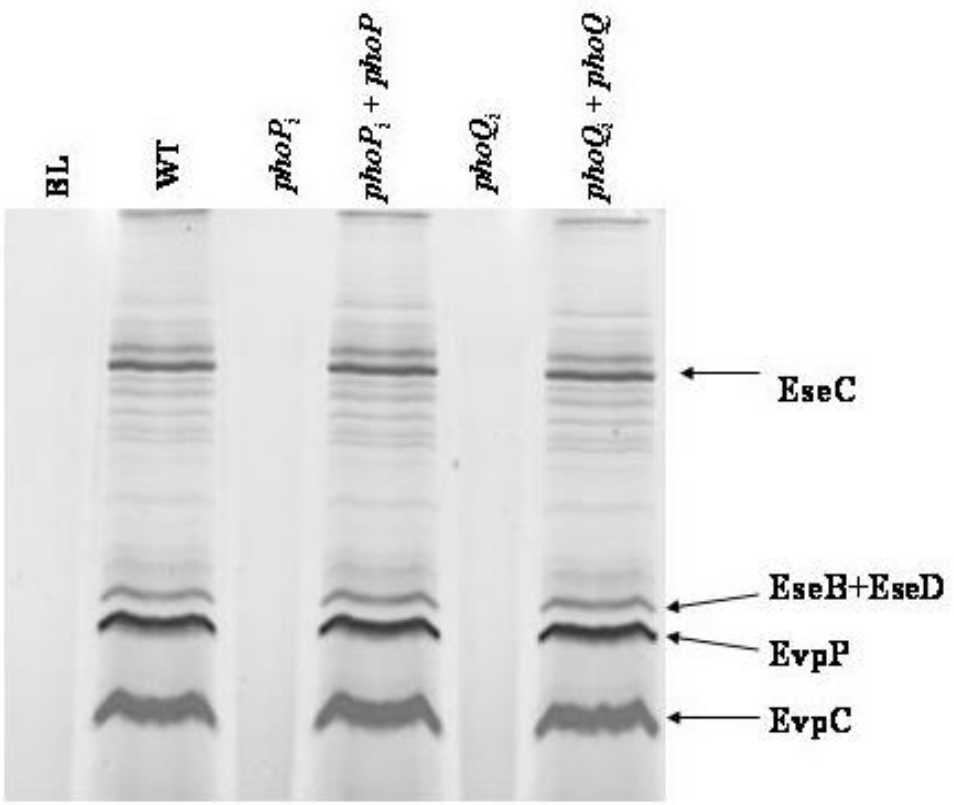

Fig. III.2 Proteome analysis of *E. tarda* 130/91 and *phoP*$_i$ and *phoQ*$_i$ mutants. Silver stained SDS-PAGE showing the ECP (T3SS: EseB, EseC and EseD; T6SS: EvpC and EvpP) secretion profiles of *E. tarda* PPD130/91 (WT), *E. tarda* carrying an insertion mutation in *phoP* (*phoP*$_i$) or *phoQ* (*phoQ*$_i$) and the mutant *E. tarda* with complementation of the corresponding wild type gene (*phoP*$_i$ + *phoP* or *phoQ*$_i$ + *phoQ*). The incubation temperature was 35^{o}C. The lane "BL" contains a blank control with only DMEM and no bacterial cells.

structures of the PhoQ sensor from *E. coli* and *S. typhimurium*, suggesting that it may also assume a PAS fold.

III.3.2 PhoP-PhoQ positively regulates T3SS and T6SS

To investigate a possible contribution of the PhoP-PhoQ system to the virulence of *E. tarda*, two *E. tarda* mutants having insertions at *phoP* (*phoP*$_i$) and *phoQ* (*phoQ*$_i$), respectively, were constructed. The growth and protein secretion profiles of the wild type PPD130/91, *phoP*$_i$, and *phoQ*$_i$ were measured at the optimal growth temperature of 35^{o}C. SDS-PAGE analysis showed that both the *phoP*$_i$ and *phoQ*$_i$ mutants were deficient in the production of extracellular proteins (ECPs), including EseB, EseC and EseD from T3SS as well as EvpC and EvpP from T6SS. At 35°C, the ECPs secreted by the wild type strain reached a concentration of 2.70 ± 0.12 μg/ml (*n*=3) after 24 h (Fig. III.2). Significantly lower levels of ECPs were observed in the *phoP*$_i$ (0.70 ± 0.03 μg/ml, *n*=3) and *phoQ*$_i$ (0.95 ± 0.11 μg/ml, *n*=3) mutants. Cell densities of the mutants were comparable to that of the wild type bacteria, suggesting that the reduced protein secretion in the mutants was not due to growth deficiencies. Complementation of *phoP*$_i$ and *phoQ*$_i$ in *trans* with a plasmid-borne wild type copy of *phoP* and *phoQ*, respectively, restored the secretion of ECPs to levels comparable to that of the wild type bacteria (Fig. III.2). These results indicated that both *phoP* and *phoQ* positively regulate the secretion of proteins from T3SS and T6SS in *E. tarda* PPD130/91.

III.3.3. PhoP binds to the promoter region of *esrB*.

Based on the previous observation that an *esrB* mutant of *E. tarda* showed an ECP profile similar to that of either the *phoP*$_i$ or *phoQ*$_i$ mutant (Zheng et al, 2005b), we speculated that PhoP regulates the secretion of T3SS and T6SS proteins by modulating the expression of EsrB.

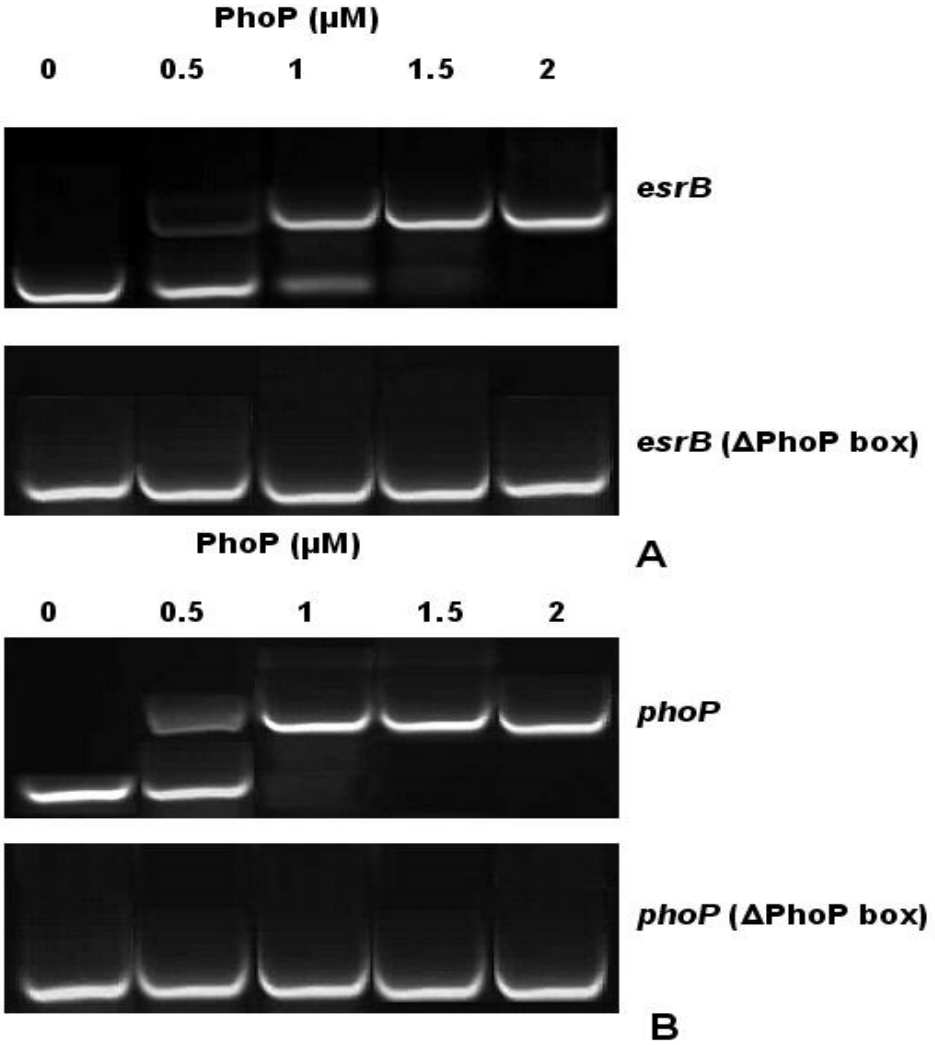

Fig. III.3. Electrophoretic mobility shift assay of PhoP binding to DNA fragments. (A)Electrophoretic mobility shift assay of PhoP protein on a 5' 6-FAM labeled DNA fragment (470 bp, from nt -467 to +3) from the promoter region of *esrB* (upper panel) and another DNA fragment with the same boundaries but with the PhoP box (nt -311 to -295) removed (lower panel). (B) Electrophoretic mobility shift assay of PhoP protein on a 5' 6-FAM labeled DNA fragment (454 bp, from nt -451 to +3) from the promoter region of *phoP* (upper panel) and another DNA fragment with the same boundaries but with the PhoP box (nt -192 to -176) removed (lower panel).

To confirm this hypothesis, the interaction of PhoP with the promoter region of *esrB* was examined. A sequence motif comprising a repeat of two hexanucleotide sequences (T/G)GTTTA separated by five bases has been defined as the high affinity PhoP binding region in *E. coli* and was named the "PhoP box" (Kato et al, 1999; Minagawa et al, 2003). Inspection of the DNA sequence upstream of *esrB* revealed a putative PhoP box of the sequence 5'-ACTCCA AAGGG CATTTA-3' between nt -311 and -295 upstream of the start codon of *esrB*. This sequence is considered to be an imperfect but orthodox match to the consensus PhoP box sequence. Using DNase I footprinting, a similar imperfect but orthodox PhoP box of the sequence 5'-TATTGA GGAGG CATTGA-3' has been identified between nt -42 and -26 relative to the PhoP-induced transcriptional start site of the *orgBC* promoter in *S. typhimurium* (Aguirre et al, 2006). Electrophoretic mobility shift assay showed that the His-tagged PhoP can bind to a 470-bp DNA fragment (from nt -467 to +3) derived from the promoter region of *esrB* containing a putative PhoP box. No interaction could be observed between PhoP and a DNA fragment derived from the same promoter region of *esrB* but with the PhoP box removed (Fig. III.3A). Inspection of the promoter region of the *phoP-phoQ* genes also revealed a putative PhoP box, with the sequence 5'-AGTTTA CCTAC CGTTGA-3', located upstream between nt -192 and -176. Electrophoretic mobility shift assay showed that PhoP can also bind to a 454-bp DNA fragment (from nt -451 to +3) derived from the promoter region of *phoP* and at an affinity even higher than that between PhoP and the promoter region of *esrB* (Fig. III.3B). Transcriptional autoregulation was also observed in the *phoPQ* operon of *S. typhimurium* (Soncini et al, 1995).

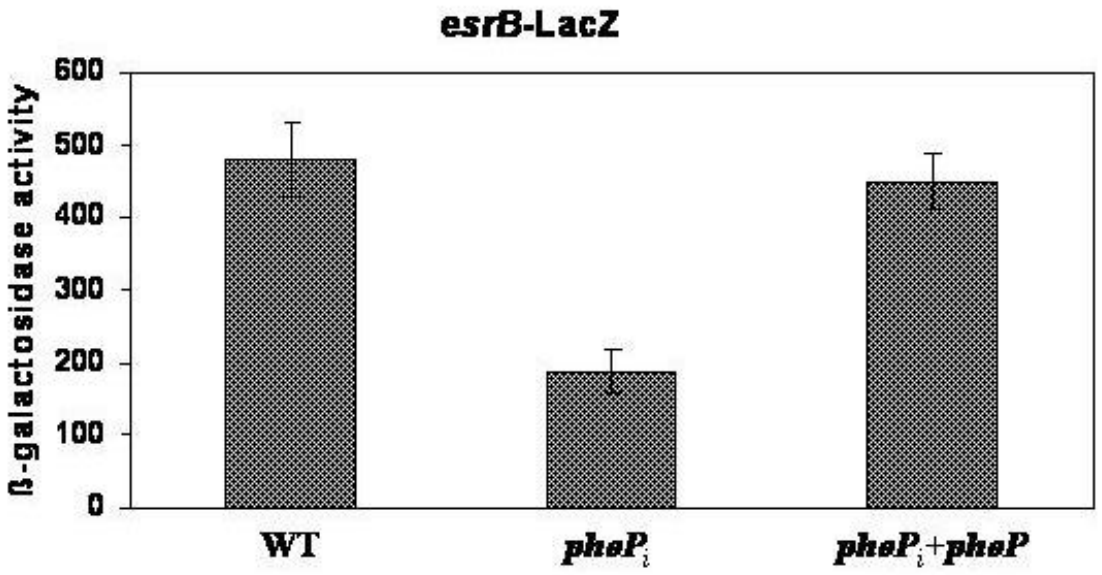

Fig. III.4 Levels of transcription of reporter strain *esrB*-LacZ in *E. tarda* PPD130/91, *phoP*$_i$ and *phoP*$_i$ + *phoP* strains. Transcription levels of the *esrB*-LacZ reporter gene fusion measured by β-galactosidase activity from bacterial cells cultured at 30^{o}C in TSB medium. WT: wild type *E. tarda* PPD130/91; *phoP*$_i$: *E. tarda* with insertion mutation in the *phoP* gene; and *phoP*$_i$ + *phoP*: *E. tarda phoP*$_i$ mutant complemented with wild type *phoP*. The values represent the mean ± SD from one representative experiment performed with triplicate samples. Equivalent results were obtained at least in triplicates.

III.3.4 PhoP regulates through *esrB*

To confirm that the expression of the *esrB* gene was controlled by PhoP, the activity of the β-galactosidase reporter genes (LacZ) for the *esrB* promoter region was measured in both the wild type strain and the *phoP*$_i$ mutant. The *esrB*-LacZ activity for the *phoP*$_i$ mutant was significantly reduced compared with that for the wild type. When the pACYC184 plasmid carrying the wild type *phoP* gene was introduced into the *phoP*$_i$ strain for complementation, the β-galactosidase activity recovered to a level similar to that of the wild type (Fig. III.4). These results indicated that PhoP, a global regulator that binds upstream of *esrB*, controls the transcription of *esrB* by modulating its promoter activity.

III.3.5 Secretion of T3SS and T6SS proteins by *E. tarda* is highly temperature dependent

To gain insight into the effects of temperature and Mg^{2+} concentration on the secretion of proteins by *E. tarda*, wild type bacterial cultures were grown at different temperatures and Mg^{2+} concentrations in TSB, and the identities and amounts of secreted ECPs were investigated. SDS-PAGE analysis showed that both T3SS (EseB, EseC and EseD) and T6SS (EvpC and EvpP) proteins were secreted by *E. tarda*. The secretion of these ECPs is prominent at growth temperatures ranging from 23^{o}C to 35^{o}C (3.00 ± 0.12 μg/ml at 23^{o}C; 3.25 ± 0.15 μg/ml at 25^{o}C; 3.00 ± 0.19 μg/ml at 30^{o}C; and 2.75 ± 0.10 μg/ml at 35^{o}C) but significantly reduced at 20^{o}C (0.80 ± 0.09 μg/ml) and 37^{o}C (1.20 ± 0.06 μg/ml). The result clearly showed that protein secretion by *E. tarda* exhibits a highly temperature dependent manner at which optimal secretion level is observed when the growth temperature is ranged between 23^{o}C to 35^{o}C, but shut down almost completely when there is only a 2-3^{o}C shift above or below this range (Fig. III.5).

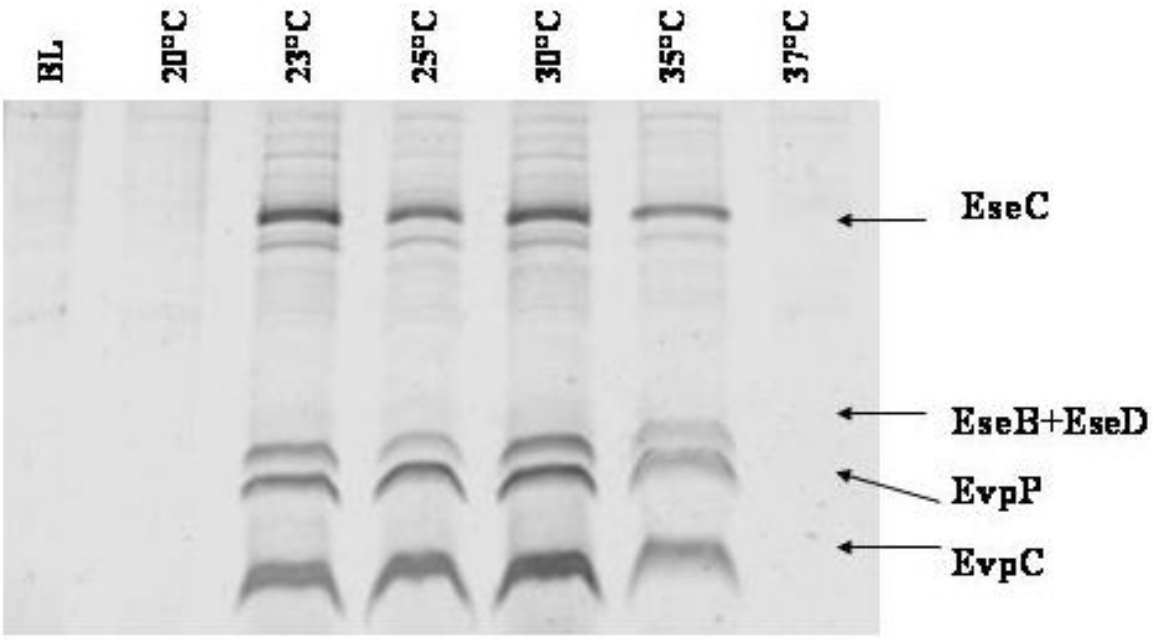

Fig. III.5 Proteome analysis of *E. tarda* 130/91 cultured at different incubation temperatures. Silver stained SDS PAGE showing T3SS and T6SS ECPs secretion by *E. tarda* PPD130/91 (cultured in DMEM for 24 hrs) at different incubation temperatures as indicted above the lanes. Lane "BL" stands for blank control with only DMEM but without bacterial cells. The ECP fractions were adjusted for equal number of bacterial cells as determined by standard plate count.

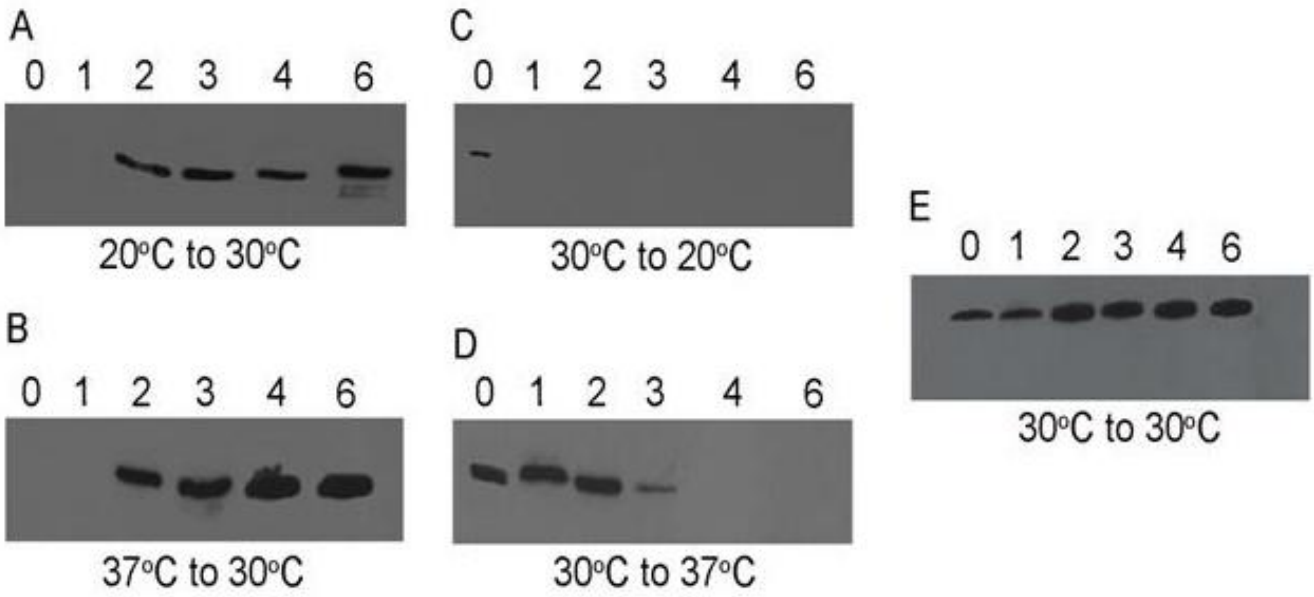

Fig. III.6 Kinetics of secretion of T3SS protein EseB at different temperatures in *E. tarda* PPD130/91. *E. tarda* was grown in TSB medium at (A) 20°C or (B) 37°C until the O.D. at 550 nm reached 0.7. The medium was then changed to fresh TSB at 30°C and the growth was continued. Alternatively, *E. tarda* was grown at 30°C until O.D. at 550 nm reached 0.7 before the medium was changed to fresh TSB at (C) 20°C or (D) 37°C. (E) Control using bacterial cells shifted from a culture at 30°C to fresh medium at the same temperature. ECPs samples were taken at 0, 1, 2, 3, 4 and 6 hrs after the change of medium and analyzed by immunoblotting using antibody against EseB from T3SS.

III.3.6. Kinetics of ECPs secretion of T3SS protein EseB by *E. tarda* at different temperatures

As the protein secretion by *E. tarda* is highly sensitive to small changes in temperature, we further studied the effect of temperature on the kinetics of EseB secretion. *E. tarda* was first grown at the optimal secretion temperature of 30^{o}C. Bacteria were harvested after reaching exponential growth phase and washed. Fresh medium was added and the incubation was continued at the secretion suppressing temperature of 20^{o}C or 37^{o}C. Samples were taken at indicated time intervals and Western blot analysis was carried out using antibodies specific for EseB, a known secreted translocator of the T3SS of *E. tarda*. Alternatively, *E. tarda* was first grown at 20^{o}C or 37^{o}C and then the incubation temperature was changed to 30^{o}C. A similar approach has been used to study the kinetics of secretion of SseB and SseC by the T3SS of *S. typhimurium* at different pH (Nikolaus et al, 2001a). Our results showed that EseB secretion by *E. tarda* could be recovered quickly and with similar length of time (1-2 hrs) when transferred from the suppressing temperature of 20^{o}C or 37^{o}C to 30^{o}C, although the time required are still slightly longer than that of the control (Figs. III.6A and III.6B). Surprisingly, the time required to suppress protein secretion differs significantly at different temperatures. The suppression of protein secretion was almost instant and took less than 1 hr when the *E. tarda* cells were transferred from 30^{o}C to 20^{o}C (Figs. III.6C); however, it took more than 3 hrs to completely suppress the secretion when the cells were transferred from 30^{o}C to 37^{o}C (Fig. III.6D).

III.3.7. *phoP/phoQ* are co-transcribed in the same promoter

Genetic organization shows the possibility of co-transciption of *phoP* and *phoQ* in the same promoter (Fig. III.7A). To confirm, RT-PCR experiments were performed using RNA isolated from *E. tarda* PPD130/91. Agarose gel electrophoresis showed that only pair B and pair C

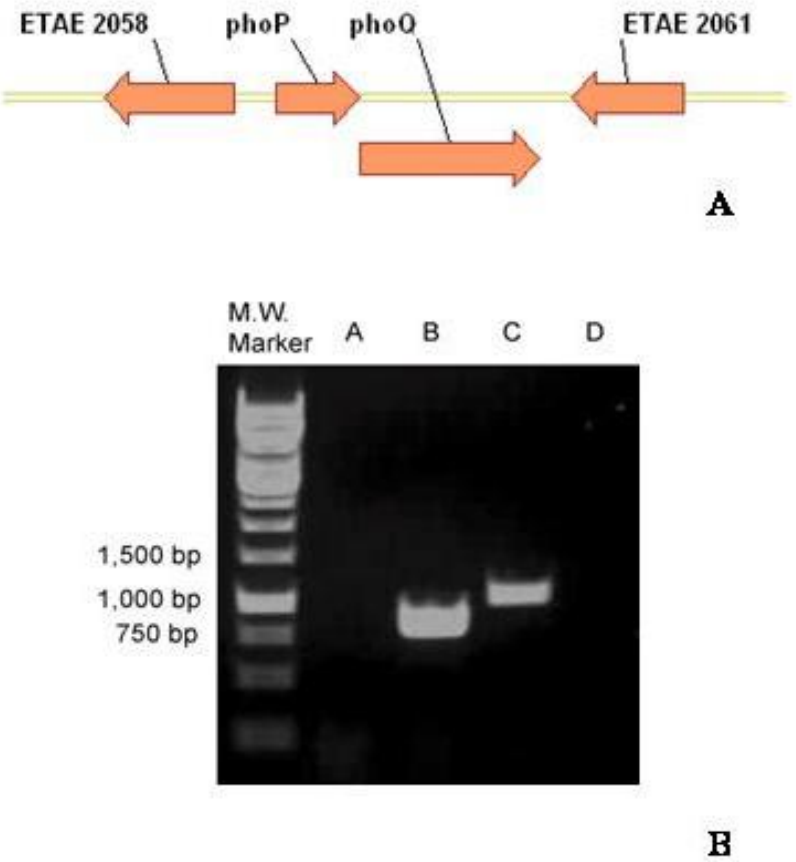

Fig. III.7. Genetic organization and co-transcription of *phoP*/*phoQ* as a single operon. (A)Genetic organization of *phoP* and *phoQ* operon. (B) RT-PCR experiments were performed using RNA isolated from *E. tarda* PPD130/91 and four pairs of primers were designed based on the genomic DNA sequence. Pair A was designed to amplify region from amino acid residue 147 of ETAE 2058 to residue 63 of PhoP; pair B from residue 36 of PhoP to residue 91 of PhoQ; pair C from residue 36 of PhoP to residue 179 of PhoQ; and pair D from residue 11 of PhoQ to residue 134 of ETAE 2061.

produced RT-PCR bands of the correct sizes (836 bp and 1,100 bp, respectively), the other two pairs carrying a portion of the adjacent genes failed to produce any RT-PCR band (Fig. III.7B). This suggests that *phoP* and *phoQ* are co-transcribed as an operon and the adjacent genes, ETAE 2058 and ETAE 2061, likely use different promoters.

III.3.8. PhoQ senses both temperature and Mg^{2+} to regulate EsrB expression

To verify the effects of temperature and Mg^{2+} concentration on the PhoP activation of *esrB*, an *esrB*-LacZ Reporter gene fusion (pRW*esrB*$_{130/91}$) under the control of the putative *esrB* promoter (from nt -900 to +150) was created, and the β-galactosidase activities of the strain were measured under different temperatures in the presence of high (10 mM) or low (1 mM) concentrations of Mg^{2+} using N-minimal medium. The expression levels of *esrB*-LacZ were approximately 30%-40% higher at the low Mg^{2+} concentration when compared with those at the high Mg^{2+} concentration (Fig. III.8A). The difference is even more significant at temperatures that suppress the ECP secretion (e.g., 60% higher at 20^{o}C and 80% higher at 37^{o}C). In general, the expression levels of *esrB*-LacZ were significantly higher for the temperature range of 23°C to 35°C when compared with those at 20°C and 37°C (Fig. III.8A). This result is in agreement with the *E. tarda* ECP secretion profiles observed at these respective temperatures and Mg^{2+} concentrations and further confirms that EsrB is a positive regulator of protein secretion by T3SS and T6SS in *E. tarda*. In addition to EsrB, the effects of temperature and Mg^{2+} concentration on the expression levels of PhoP was studied using a new strain harboring lacZ fusion genes under the control of the putative promoters for *phoP* (from nt -1460 to +150) (Fig. III.8B). LacZ transcriptional fusion with the PhoP (pRW*phoP*$_{130/91}$) protein showed temperature and Mg^{2+} concentration responsive phenomena similar to those seen for pRW*esrB*$_{130/91}$.

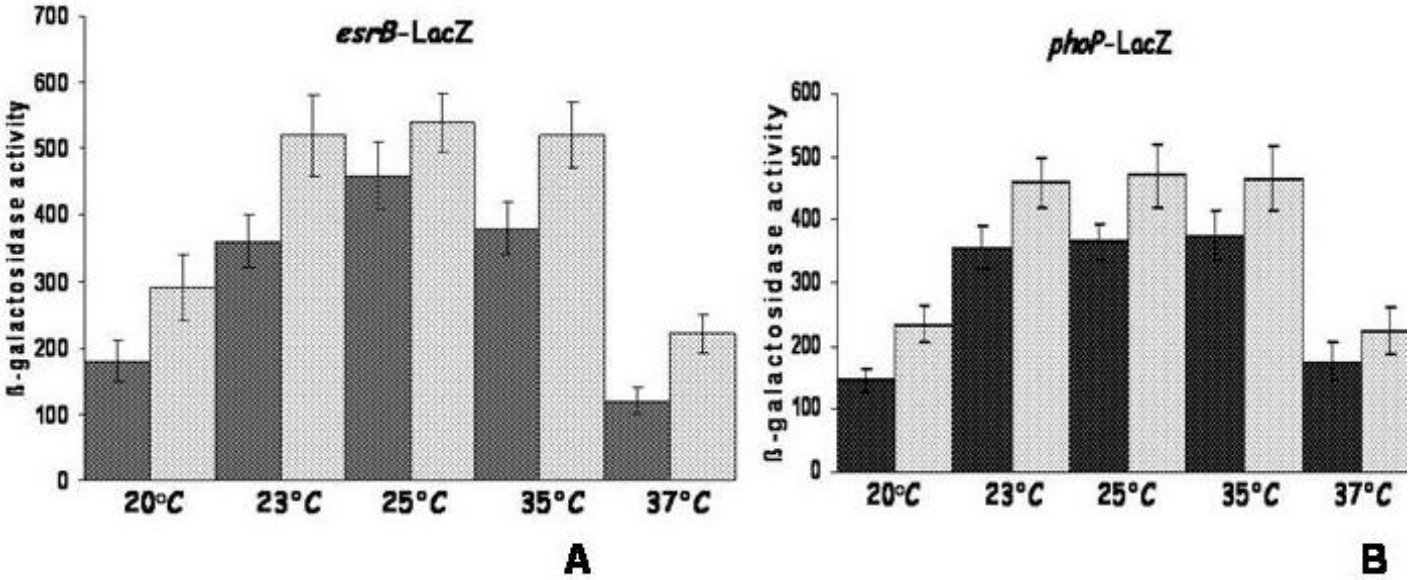

Fig. III.8. Effect of temperature on the expression of *esrB* and *phoP*. ß-galactosidase activities of reporter genes (A) *esrB*-LacZ; (B) *phoP*-LacZ are determined in N-minimal medium under different incubation temperatures in the presence of 1 mM (light grey bars) or 10 mM (dark grey bars) of Mg^{2+}. All the 3 genes showed reduced activities at 20°C and 37°C compared to 23°C, 25°C and 35°C. The activities of these genes are also repressed at a higher concentration of Mg^{2+} and the effect is additive with that of temperature. The values represent the mean ± SD from one representative experiment performed with triplicate samples. Equivalent results were obtained at least in triplicates.

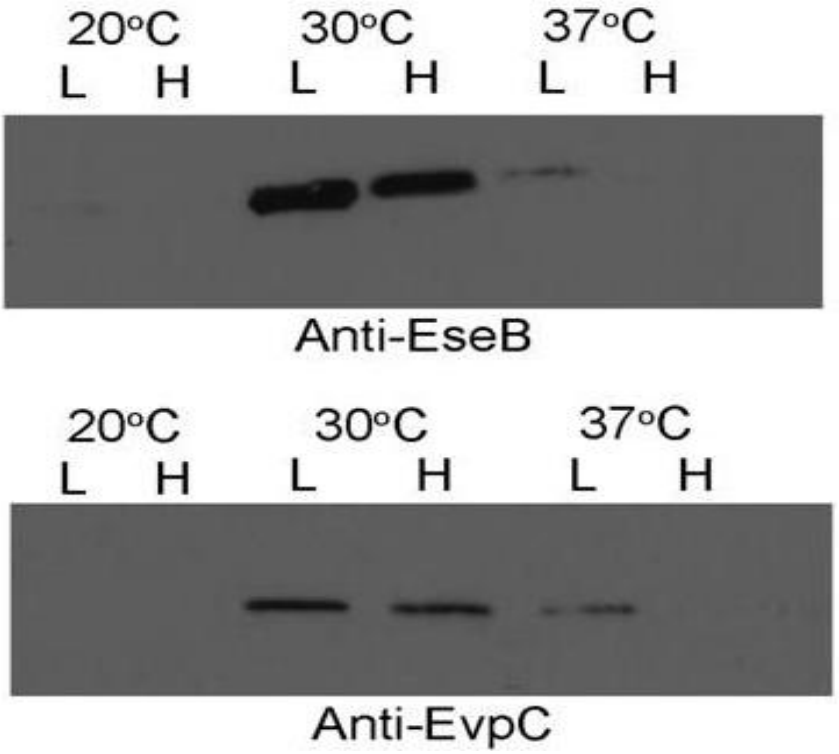

Fig. III.9. Additive effect of Mg^{2+} and temperature on the secretion of EseB and EvpC. Western blot analysis showing the secretion of EseB from T3SS (upper panel) and EvpC from T6SS (lower panel) by *E. tarda* at the temperatures of 20°C, 30°C and 37°C in the presence of 1 mM (L) or 10 mM (H) Mg^{2+} using N-minimal medium.

Other than the promoter activities, we have also compared the amount of protein secreted by the T3SS (EseB) and T6SS (EvpC) of *E. tarda* at different temperature and Mg^{2+} concentration. A clear suppression of secretion of both proteins was observed at 20°C and 37°C as compared to that at 30°C. The suppression of protein secretion is more significant in the presence of 10 mM Mg^{2+} and the effect of temperature and Mg^{2+} are additive, at least at 30°C and 37°C (Fig. III.9).

III.3.9. PhoQ sensor domain undergoes a conformational change at low temperatures

We hypothesized that PhoQ is responsible for sensing environmental temperature and Mg^{2+} concentration and that it regulates T3SS and T6SS through EsrB. To verify these hypotheses, the sensor domain of *E. tarda* PhoQ was cloned and expressed, and its thermal denaturation was studied in the presence or absence of Mg^{2+} using circular dichorism (CD). The N-terminal sensor domain of PhoQ ($PhoQ_s$) from residues 45 to 187 is located between two predicted transmembrane helices and is speculated to be periplasmic. Although the $PhoQ_s$ protein was expressed in inclusion bodies (Fig. III.10), it could be refolded properly in a buffer containing a minimal amount of 0.1 mM Mg^{2+}, as shown by CD. There was a significant decrease in the secondary structure (raw ellipticity from the CD spectra) of $PhoQ_s$ as the temperature increased from 35°C to 37°C indicating the melting of the protein at a temperature above 35°C (Fig. III.11A). However addition of 10mM Mg^{2+} did not change the profile of the Far-UV CD (Fig. III.19). Thermal denaturation followed by CD at 206.5 nm Mg^{2+} showed a sharp conformational transition between 30°C and 40°C for $PhoQ_s$ in the absence of Mg^{2+}, with a T_m of around 37.9°C after curve fitting according to the equation by Ruiz-Sanz, J. *et al* (Ruiz-Sanz et al, 1995).

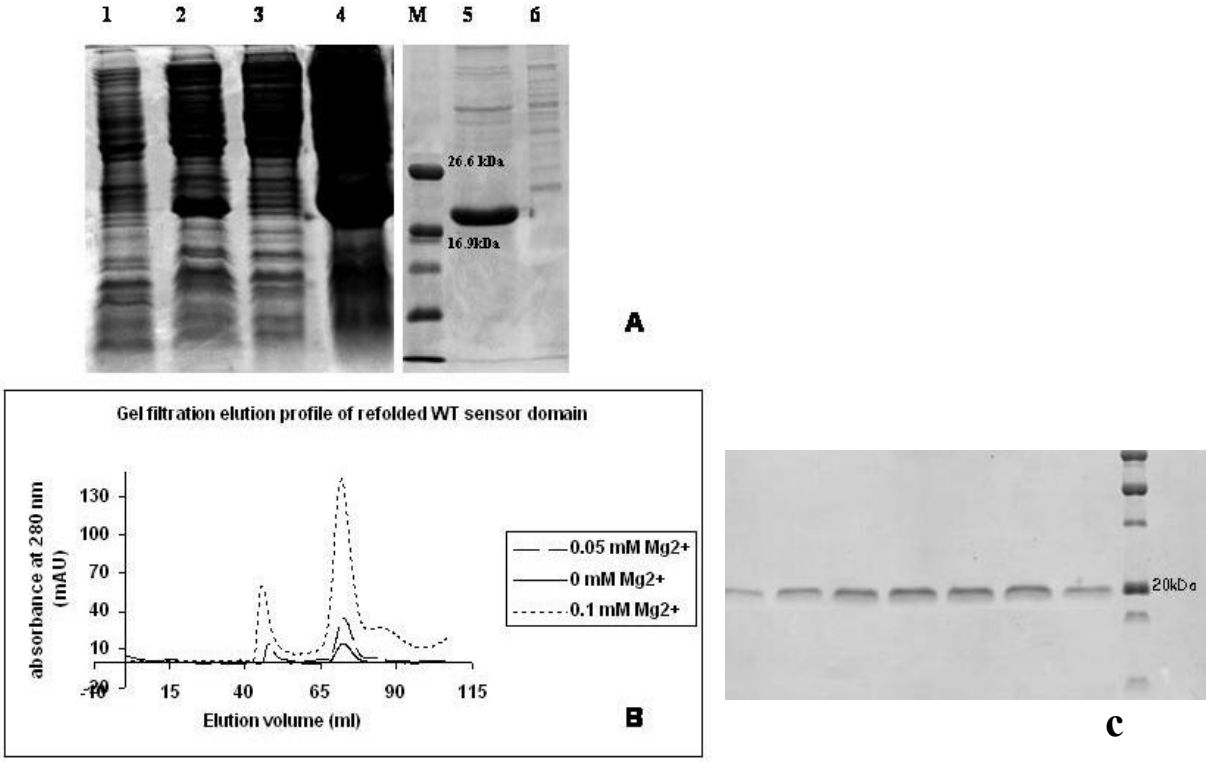

Fig. III.10. Expression and purification of His-tag PhoQ sensor domain. (A) PhoQ sensor domain was expressed as an insoluble protein in *E.coli* BL-21 cells. Lane 1, whole cell before IPTG induction; lane 2, whole cell after IPTG induction; lane 3, soluble fraction after sonication, lane 4, pellet fraction after sonication, lane 5, eluted protein from Ni-NTA column; lane 6,flow through from Ni-NTA affinity column and lane M, low molecular weight protein marker. (B) FPLC gel filtration profile of PhoQ sensor domain on Superdex 75pg column refolded using indicated concentration of Mg^{2+}. (C) Fractions after gel filtration were run on a SDS-PAGE and pooled together for the biochemical studies.

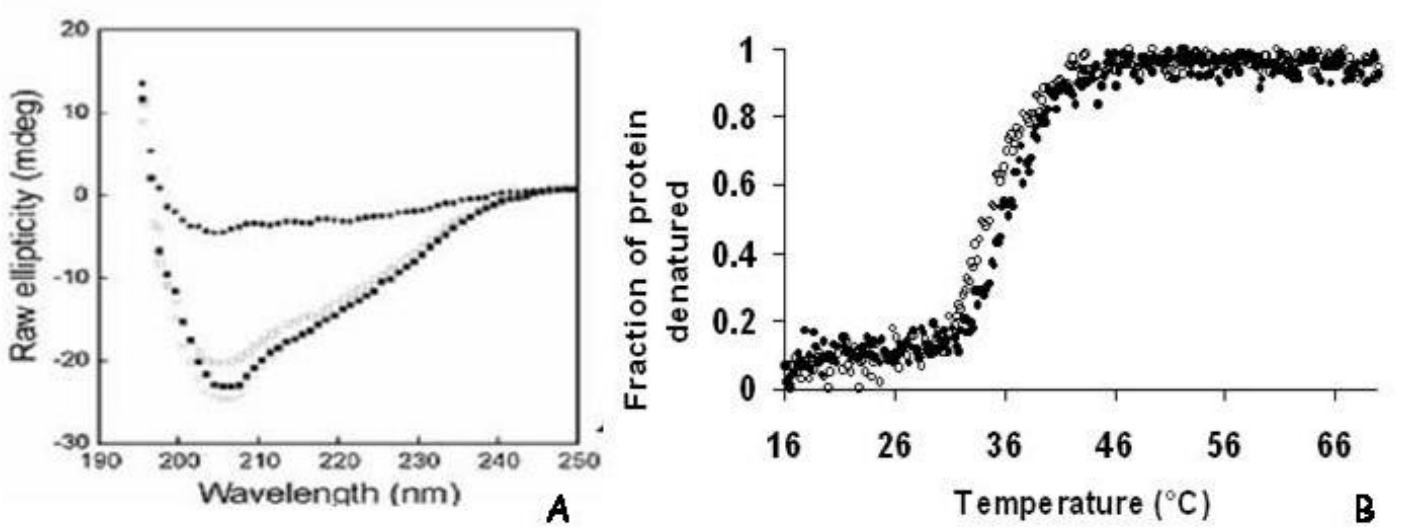

Fig. III.11. Loss of secondary structure of PhoQ sensor domain upon a temperature shift from 35°C to 37°C. (A) Far-UV CD spectra of the PhoQ sensor domain at 20°C (open circle), 30°C (closed square), 35°C (open square) and 37°C (closed circle). (B) Thermal denaturation of the *E. tarda* PhoQ sensor domain monitored by CD at 206.5 nm in the presence (closed circle) or absence (open circle) of 10 mM Mg^{2+}.

The addition of 10mM Mg^{2+} stabilized the $PhoQ_s$ only slightly, with the T_m shifting to about 40.2°C (Fig. III.11B). A thermal denaturation experiment carried out on the homologous PhoQ sensor domain from *E. coli* obtained a T_m of 59.2°C and 65.4°C in the absence and presence of 10 mM Mg^{2+}, respectively (Waldburger & Sauer, 1996). This sharp conformational transition in the $PhoQ_s$ of *E. tarda* at a relatively low temperature is consistent with the temperature transition (35°C to 37°C) that regulates the secretion of proteins by T3SS and T6SS, suggesting that this conformational change could be the mechanism employed by PhoQ to sense the environmental temperature. No conformational transition, however, was detected by CD between 16°C and 30°C, a range within which another temperature transition (20°C to 23°C) was supposed to regulate protein secretions by T3SS and T6SS in *E. tarda*.

III.3.10 PhoQ has greater stability at 30°C in comparison to 20°C and 37°C

To determine if there was any conformational change of $PhoQ_s$ at 20°C, the equilibrium urea denaturation of *E. tarda* $PhoQ_s$ was monitored by CD at 210 nm at 20°C, 30°C and 37°C in the absence or presence of 10 mM Mg^{2+}. The data was curve fitted according to the equation of Mok, Y.K. *et al* (Mok et al, 1995) to obtain the ΔG values. In the absence of Mg^{2+}, PhoQs had a higher stability towards urea at 30°C (ΔG = 8.5 kcal/mol) than at 37°C (ΔG = 7.6 kcal/mol) (Fig. III.12). In the presence of 10 mM Mg^{2+}, the stability at both 30°C (ΔG = 9.4 kcal/mol) and 37°C (ΔG = 8.6 kcal/mol) increased slightly. The lower stability of $PhoQ_s$ at 37°C towards urea, when compared with that at 30°C, is consistent with the thermal denaturation data. Interestingly, there was a similar drop in the stability of $PhoQ_s$ towards urea at 20°C (ΔG = 7.3 kcal/mol and 8.0 kcal/mol in the absence and presence of 10 mM Mg^{2+}, respectively) compared with that at 30°C, suggesting that PhoQ may also sense the temperature transition

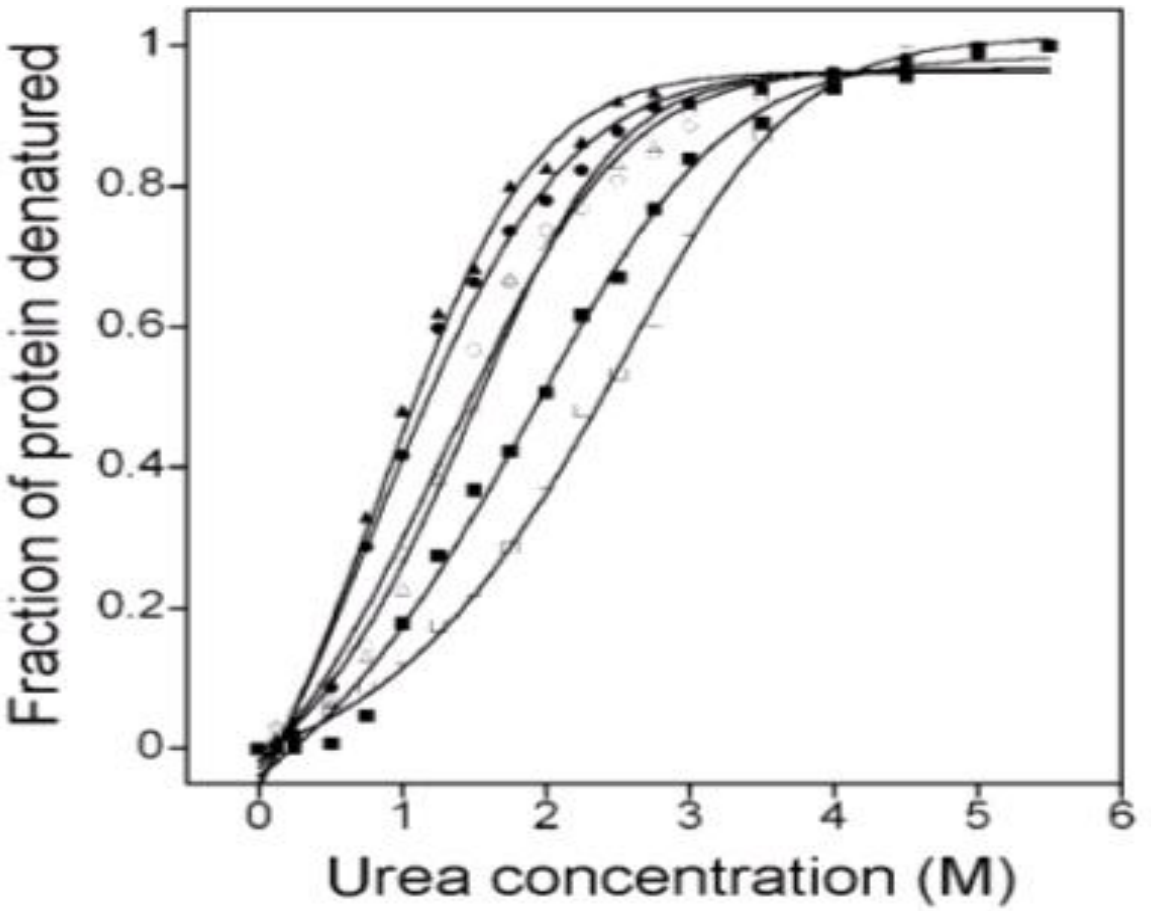

Fig. III.12. **Urea denaturation CD of PhoQ sensor domain at different temperatures.** Urea denaturation of the *E. tarda* PhoQ sensor domain monitored by CD at 210 nm at 20^{o}C (circle), 30^{o}C (square) and 37^{o}C (triangle) in the presence (open symbols) or absence (closed symbols) of 10 mM Mg^{2+}.

between 20°C and 23°C and regulate the T3SS and T6SS of *E. tarda* within this temperature range.

III.3.11. Tertiary structure of PhoQ sensor shows no significant changes to temperature

Fluorescence spectra were used to determine if there were any tertiary structure changes following the urea denaturation of $PhoQ_s$ at different temperatures in the absence or presence of 10 mM Mg^{2+}. Surprisingly, there were no significant changes in the stability of the $PhoQ_s$ tertiary structure at the temperatures of 20°C, 30°C and 37°C (Fig. III.13). The addition of 10 mM Mg^{2+} stabilized the $PhoQ_s$ domain by approximately 1-2 kcal/mol, comparable to what had been observed previously in the *E. coli* PhoQ sensor domain (Waldburger & Sauer, 1996). During urea denaturation, the secondary structure of $PhoQ_s$ starts to melt before the tertiary structure, suggesting that, in $PhoQ_s$, conformational changes due to temperature transitions mainly involve secondary structures and that the secondary structures appear to change more readily than the tertiary structure.

III.3.12. Thr and Pro residues are responsible for temperature sensing by PhoQ

To understand the mechanism underlying the temperature-dependent conformational changes of $PhoQ_s$ secondary structures, sequence of $PhoQ_s$ from *E. tarda* was compared with those from other bacteria. The results showed that some residues at the turn regions of $PhoQ_s$ of *S. typhimurium* and *E. coli* were replaced with either Pro or Thr residues in $PhoQ_s$ of *E. tarda* (Fig. III.1). Based on this observation, these Pro and Thr residues were selected for site-specific mutation studies. Six single point mutants of *E. tarda* $PhoQ_s$, (T76E, P77L, P79E, P120N, P140H and T167P) were generated, by converting selected Pro or Thr residues to the corresponding residues in *S. typhimurium* and *E. coli*. Thermal denaturation studies monitored by Far-UV CD showed that $PhoQ_s$ mutants P120N and T167P had T_m of 55.5°C and 59.0°C,

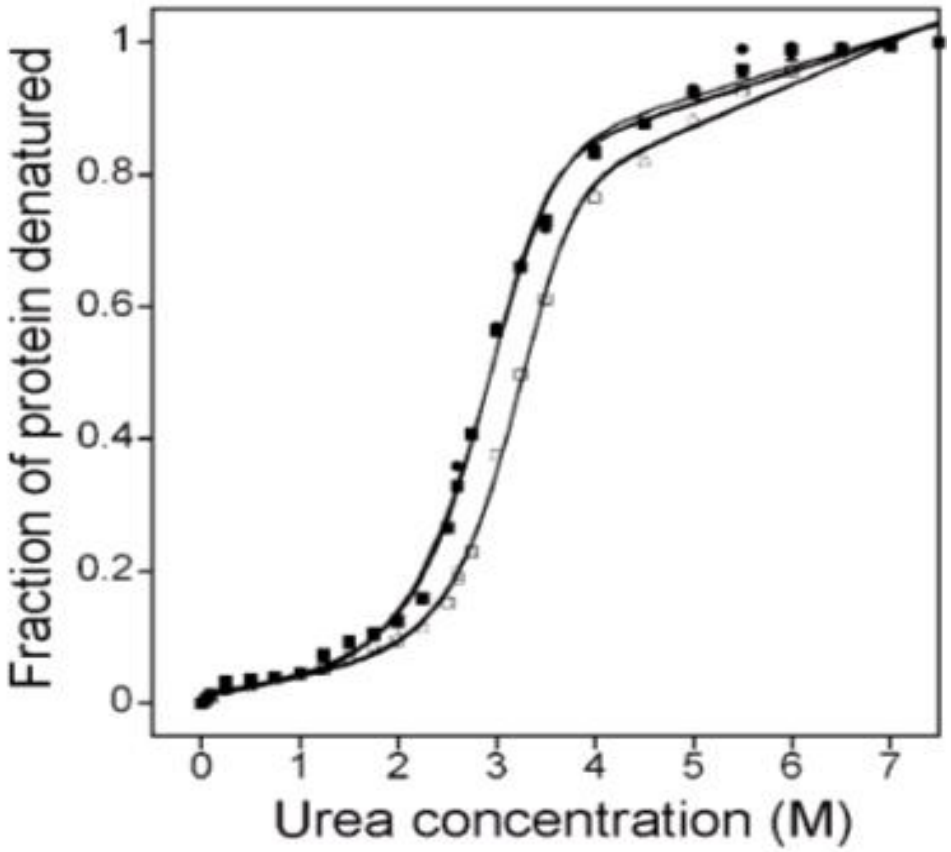

Fig. III.13. Urea denaturation fluorescence of PhoQ sensor domain. Urea denaturation of the *E. tarda* PhoQ sensor domain monitored by fluorescence at 350 nm at 20°C (circle), 30°C (square) and 37°C (triangle) in the presence (open symbols) or absence (closed symbols) of 10 mM Mg^{2+}.

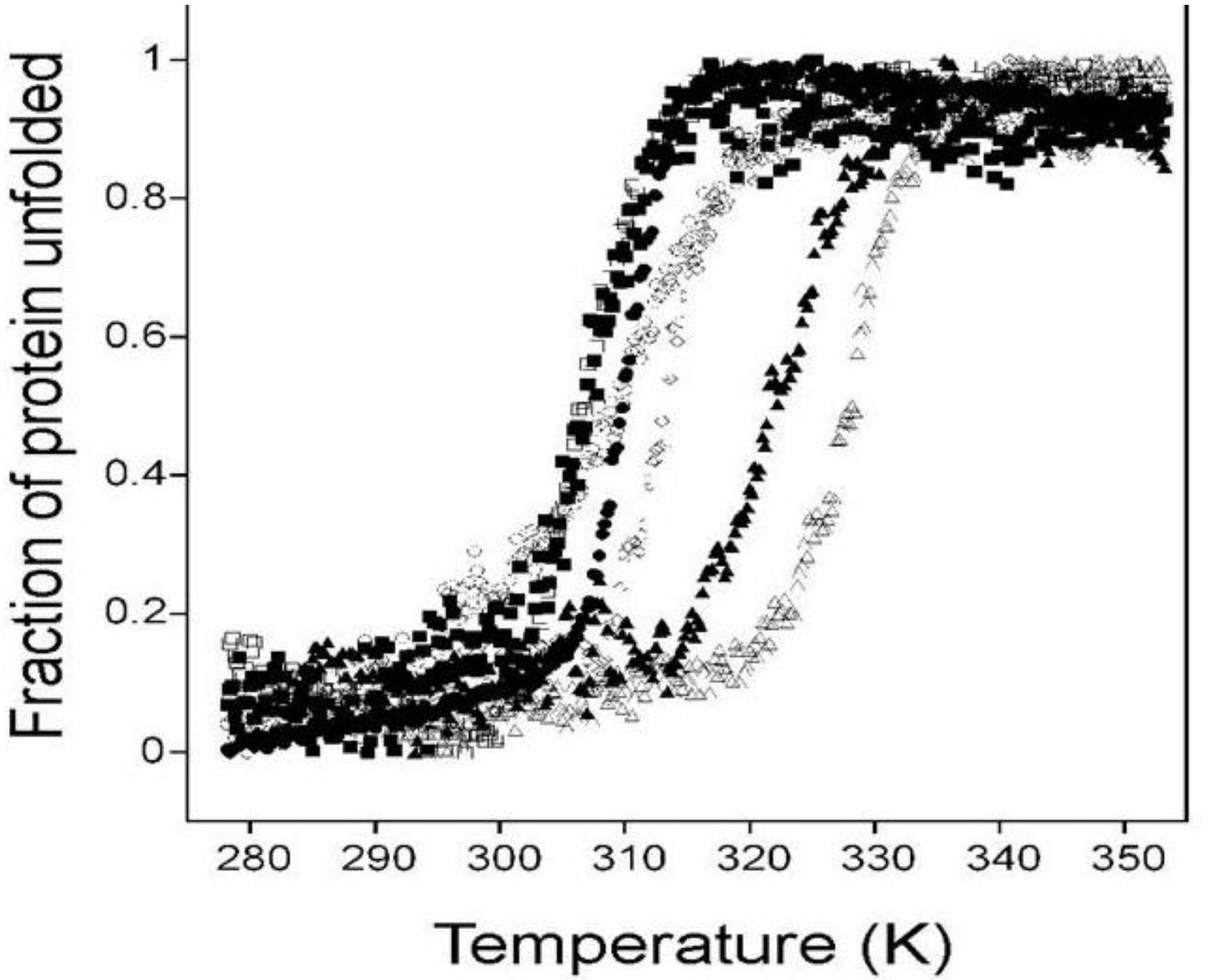

Fig. III.14. Thermal denaturation of Thr or Pro residue mutants of the *E. tarda* PhoQ sensor domain. Thermal denaturation of the wild type and mutant *E. tarda* PhoQ sensor domains monitored by CD at 202 nm for P79E (open rhombus) and P120N (closed triangle); at 206.5 nm for T167P (open triangle); and at 218 nm for wild type (open square), T76E (closed square), P77L (open circle) and P140H (closed circle). All samples had a concentration of 10 mM Mg^{2+}.

respectively, which were both significantly higher than that of the wild type $PhoQ_s$ (T_m = 37.9°C) but comparable with that of the *E. coli* PhoQ sensor domain (T_m = 65.4°C in 10 mM Mg^{2+}) (Waldburger & Sauer, 1996) (Fig. 5A). In contrast, the $PhoQ_s$ mutants T76E (T_m = 38.6°C) and P140H (T_m = 40.8°C) had T_m values similar to that of the wild type protein, and, the $PhoQ_s$ mutants P77L (T_m = 45.3°C) and P79E (T_m = 44.4°C) had T_m values slightly higher than that of the wild type protein (Fig. III.14). The ΔH_m value (related to the slope of transition) of P77L (6.5 x 10^4 kcal/mol), however, was much smaller than that of P79E (1.4 x 10^5 kcal/mol).

III.3.13. Mutant T167P is stable and shows no such temperature transition response unlike the wild type protein.

Analysis of the urea stability (monitored by CD at 210 nm) of the most thermally stable mutant, T167P, revealed that, unlike the wild type protein, temperature (at 20°C, 30°C or 37°C) had no effect on its stability towards urea, suggesting that mutation of a single Pro or Thr residue within the turn region of *E. tarda* $PhoQ_s$ could abolish the temperature transition responses (20°C to 23°C and 35°C to 37°C) that occur in the wild type protein (Fig. III.15).

III.3.14. Differential behavior of mutant bacteria carrying certain point mutations in response to temperature.

Based on our hypothesis that the temperature transition responses were responsible for temperature sensing by PhoQs, the mutants P120N and T167P were predicted to lose their ability to sense temperature and affect ECP secretion through T3SS and T6SS. On the other hand, mutants T76E and P140H were predicted to exhibit little effect, whereas P77L and P79E were predicted to exhibit an intermediate effect on the temperature-regulated secretion by *E. tarda* T3SS and T6SS. To verify these predictions, the *E. tarda* strain carrying the *$phoQ_i$*

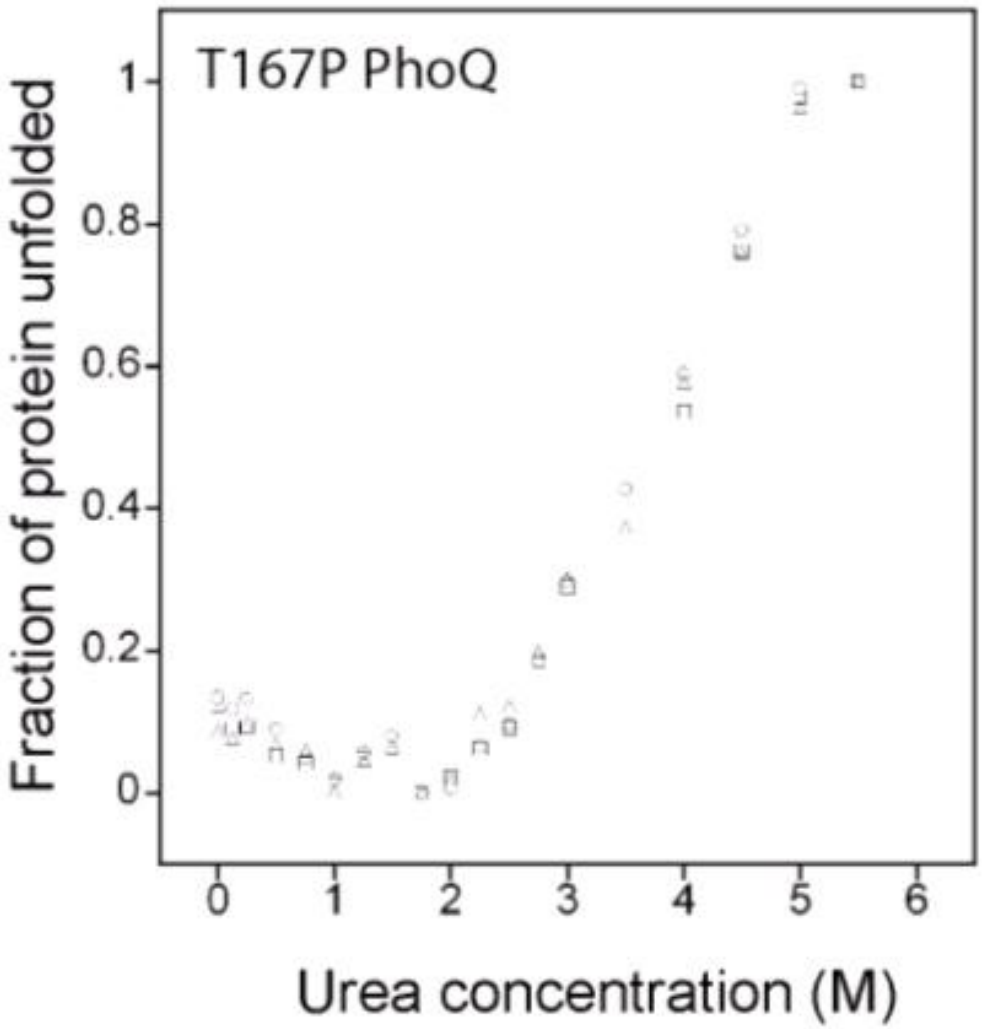

Fig. III.15. Urea denturation CD of *E. tarda* PhoQ T167P mutant sensor domain. Urea denaturation of the *E. tarda* PhoQ T167P mutant sensor domain monitored by CD at 210 nm at 20^{o}C (open circle), 30^{o}C (open square) and 37^{o}C (open triangle) in the presence of 10 mM Mg^{2+}.

insertion mutation was complemented with a full length *phoQ* gene carrying a single site mutation at various Pro and Thr residues. Complementation with the thermally stable mutants P79E, P120N and T167P could not recover the effect of $phoQ_i$, indicating that they are "loss-of-function" mutations in which the PhoQ mutant cannot activate PhoP and promote the secretion of ECPs from *E. tarda* T3SS and T6SS at any of the temperatures tested. In contrast, complementation with PhoQ mutants that have thermal stabilities similar to that of the wild type protein (e.g., T76E and P140H) could completely recover the effect of $phoQ_i$ (Fig. III.16). Interestingly, complementation with the mutant P77L rendered the *E. tarda* $phoQ_i$ strain "temperature-blind" and resulted in the constitutive secretion of ECPs at 20^oC. The PhoQ P77L mutant was, however, still functional in its ability to suppress ECP secretion at 37^oC. The PhoQ P77L mutant had a T_m value (45.3^oC) close to that of the "loss-of-function" P79E mutant but with a much lower ΔH_m value (6.5 x 10^4 kcal/mol), suggesting that these two mutants could respond differently to changes in temperature (Fig. III.16).

III.3.15. Periplasmic sensor domain of *E. tarda* PPD130/91 PhoQ is responsible for the unique temperature transition phenomenon unlike homologous bacteria EPEC 2348/69.

To confirm that *E. tarda* PPD130/91 PhoQ sensor domain is responsible for the unique temperature sensing activity, the amount of ECPs from *E. tarda* $phoQ_i$ mutant complemented with the *phoQ* gene from EPEC 2348/69 was determined. As a human pathogen, EPEC 2348/69 secreted translocators, such as EspA, EspB and EspD, from the T3SS at 37^oC but not at 30^oC (Li et al, 2004). The *E. tarda* $phoQ_i$ mutant, when complemented with *phoQ* from EPEC 2348/69, secreted similar levels of ECPs at 37^oC (2.7 ± 0.07 μg/ml) as compared to the wild type *E. tarda* at 30^oC (Fig. III.17 and Fig. III.18). At 30^oC, a much reduced amount of ECPs was secreted (1.4 ± 0.06μg/ml) by this mutant.

Strains	ECP (µg/ml) 20°C	35°C	37°C	T_m (°C)	ΔH_m (cal/mol)
WT	0.80 ± 0.02	2.70 ± 0.12	1.20 ± 0.03		
phoQ$_i$	0.71 ± 0.03	0.95 ± 0.11	0.90 ± 0.02		
phoQ$_i$ + *phoQ* WT	0.72 ± 0.02	2.56 ± 0.15	1.10 ± 0.01	37.9	1.1×10^5
phoQ$_i$ + *phoQ* T76E	1.00 ± 0.07	2.40 ± 0.05	0.81 ± 0.06	38.6	1.2×10^5
phoQ$_i$ + *phoQ* P77L	2.50 ± 0.06	2.60 ± 0.02	1.00 ± 0.08	45.3	6.5×10^4
phoQ$_i$ + *phoQ* P79E	0.67 ± 0.05	1.00 ± 0.10	0.95 ± 0.04	44.4	1.4×10^5
phoQ$_i$ + *phoQ* P120N	0.70 ± 0.03	1.10 ± 0.09	0.98 ± 0.03	55.5	1.0×10^5
phoQ$_i$ + *phoQ* P140H	0.80 ± 0.04	2.45 ± 0.06	0.98 ± 0.02	40.8	1.5×10^5
phoQ$_i$ + *phoQ* T167P	0.53 ± 0.07	0.70 ± 0.03	0.65 ± 0.04	59.0	1.5×10^5

Fig. III.16. Amount of ECP obtained at the incubation temperatures of 20°C, 35°C and 37°C from wild type *E. tarda*, the *E. tarda phoQ*$_i$ mutant, the *E. tarda phoQ*$_i$ mutant complemented with various *phoQ* gene mutations. The T_m and ΔH_m values obtained from the curve fitting of the thermal denaturation data from the wild type and mutant PhoQs are also listed.

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PhoQ_ET          SFDKTTYRLLRGESNLFFSLAHWQDGRLTITPPPNMALNTPTLVMIYDREGRLLWSQRQV
PhoQ_EPEC        SFDKTTFRLLRGESNLFYTLAKWENNKLHVELPENIDKQSPTMTLIYDENGQLLWAQRDV
                 ******:**********::**:*::.:* :  * *:  ::**:.:***.:*:***:**:*
PhoQ_ET          PALEKRIARSWLEKPGFYEIDSNKHFSSTVLGDNPQMQDQLKDY-DDDSADAMTHSVAVS
PhoQ_EPEC        PWLMKMIQPDWLKSNGFHEIEADVNDTSLLLSGDHSIQQQLQEVREDDDDAEMTHSVAVN
                 * * * *  .**:. **:**::: : :* :*..: .:*:**::  :**.   *******.
PhoQ_ET          QYTATAHLPALTIVVVDTIPQELQNSDM
PhoQ_EPEC        VYPATSRMPKLTIVVVDTIPVELKSSYM
                  *.**:::* ********** **:.* *
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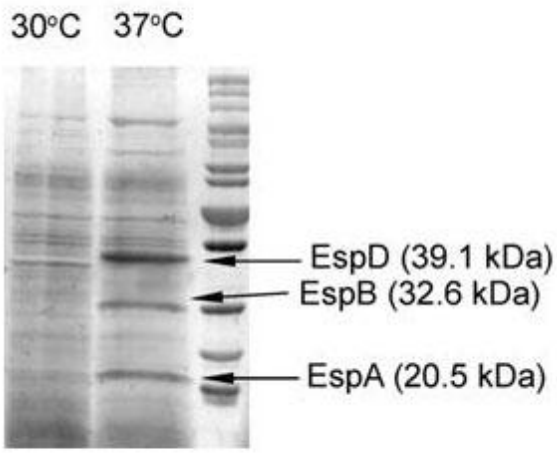
A



B

Fig. III.17. ECP secretion and sequence alignment of *EPEC* PhoQ sensor domain with *E. tarda* 130/91. (A) Sequence alignment of PhoQ sensor domain from *E. tarda* PPD130/91 with that from EPEC 2348/69. Pro and Thr residues that are not conserved between the two proteins are highlighted. (B) ECP assay showing secretion of proteins from the T3SS of EPEC 2348/69 only at 37°C but not at 30°C.

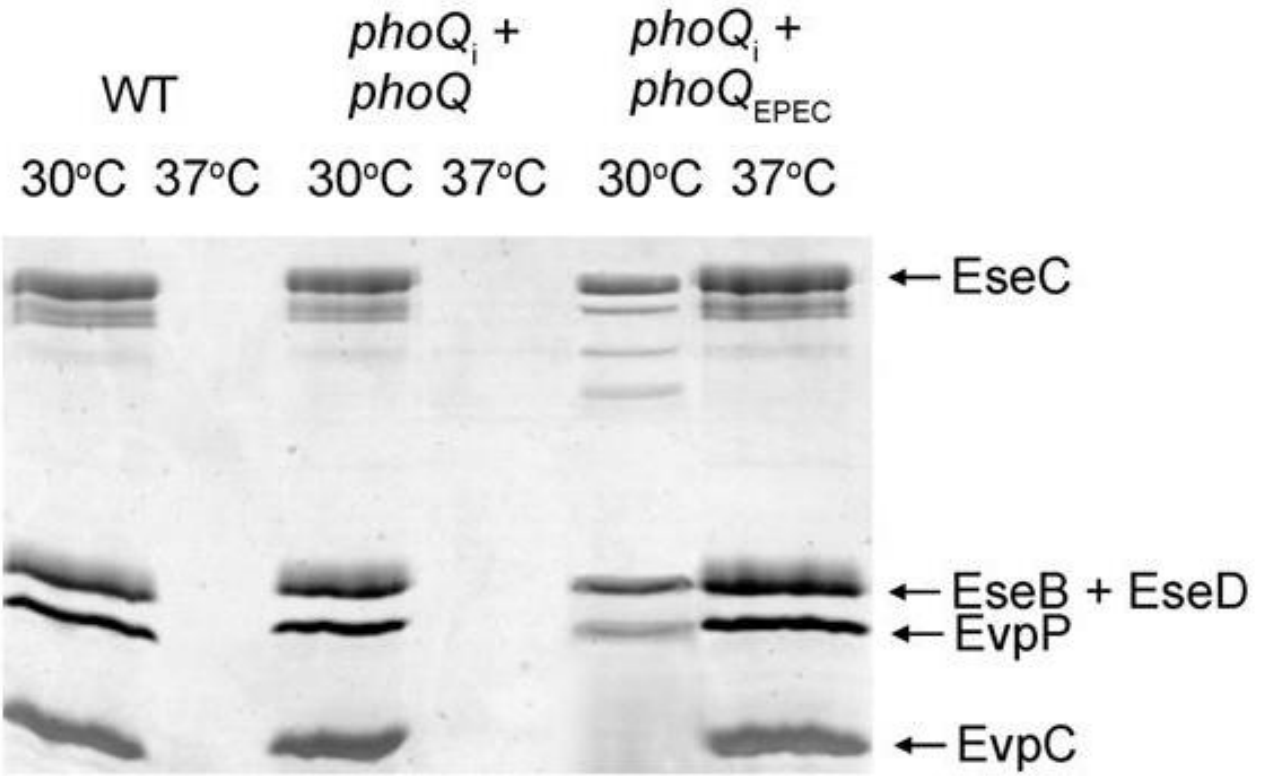


Fig. III.18. ECP secretion of *E. tarda* PPD 130/91 and the complemented mutant strains. ECP assay showing secretion of proteins from the T3SS and T6SS of wild type *E. tarda* and *E. tarda phoQ*$_i$ mutant complemented with *phoQ* only at 30°C (2.2 ± 0.06 μg/ml) but not at 37°C (0.35 ± 0.04 μg/ml). In contrast, *E. tarda phoQ*i mutant complemented with *phoQ* from EPEC secreted similar levels of proteins at 37°C (2.7 ± 0.07 μg/ml) as wild type *E. tarda* at 30°C (2.5 ± 0.09 μg/ml). At 30°C, the levels of proteins secreted by the *E. tarda phoQ*$_i$ + *phoQ*$_{EPEC}$ mutant were significantly reduced (1.4 ± 0.06 μg/ml), especially for proteins from the T6SS.

III.3.16. The PhoQ sensor binds Mg^{2+}

As our results showed that Mg^{2+} and temperature had additive effects on the regulation of *E. tarda* T3SS and T6SS, we attempted to characterize the binding of Mg^{2+} to $PhoQ_s$. The binding of Mg^{2+} to $PhoQ_s$ had no effect on the Far-UV CD spectra of the protein (Fig. III.19), similar to the observation seen for the PhoQ sensor domain of *E. coli* (Waldburger & Sauer, 1996). Therefore, the intrinsic fluorescence (excitation at 280 nm and emission at 350 nm) of the *E. tarda* $PhoQ_s$ was used instead to study the direct binding of Mg^{2+} to the protein. This assay showed that Mg^{2+} can bind directly to $PhoQ_s$ with a K_d value of approximately 95 μM (Fig. III.20B). Temperature affected the secondary structure of $PhoQ_s$ but had little effect on the affinity of Mg^{2+} binding by $PhoQ_s$ from 20°C to 37°C.

III.3.17. Mg^{2+} sensing takes place through acidic cluster residues

A previous study showed that divalent cations may bind to a cluster of acidic residues (EDDDDAE) and stabilize PhoQ in an inactive conformation to prevent PhoP-mediated transcription in response to divalent-cation starvation *in vivo* (Waldburger & Sauer, 1996). A similar cluster of acidic residues with a slightly different sequence (DDDSADA) is also observed in the *E. tarda* $PhoQ_s$. Replacement of these acidic residues with conservative uncharged residues (NNNSANA) completely abolished Mg^{2+} binding by the *E. tarda* $PhoQ_s$, indicating that this acidic cluster is the putative Mg^{2+} binding site (Fig. III.20B). The acidic cluster mutant has a slightly higher stability than the wild type $PhoQ_s$, as monitored by CD at 218 nm (T_m = 47.4°C), which is in agreement with a similar observation for the *E. coli* PhoQ sensor domain (Fig. III.20A). Unlike the wild type *E. tarda* $PhoQ_s$, the addition of 10 mM Mg^{2+} did not provide additional stabilization to the acidic cluster mutant.

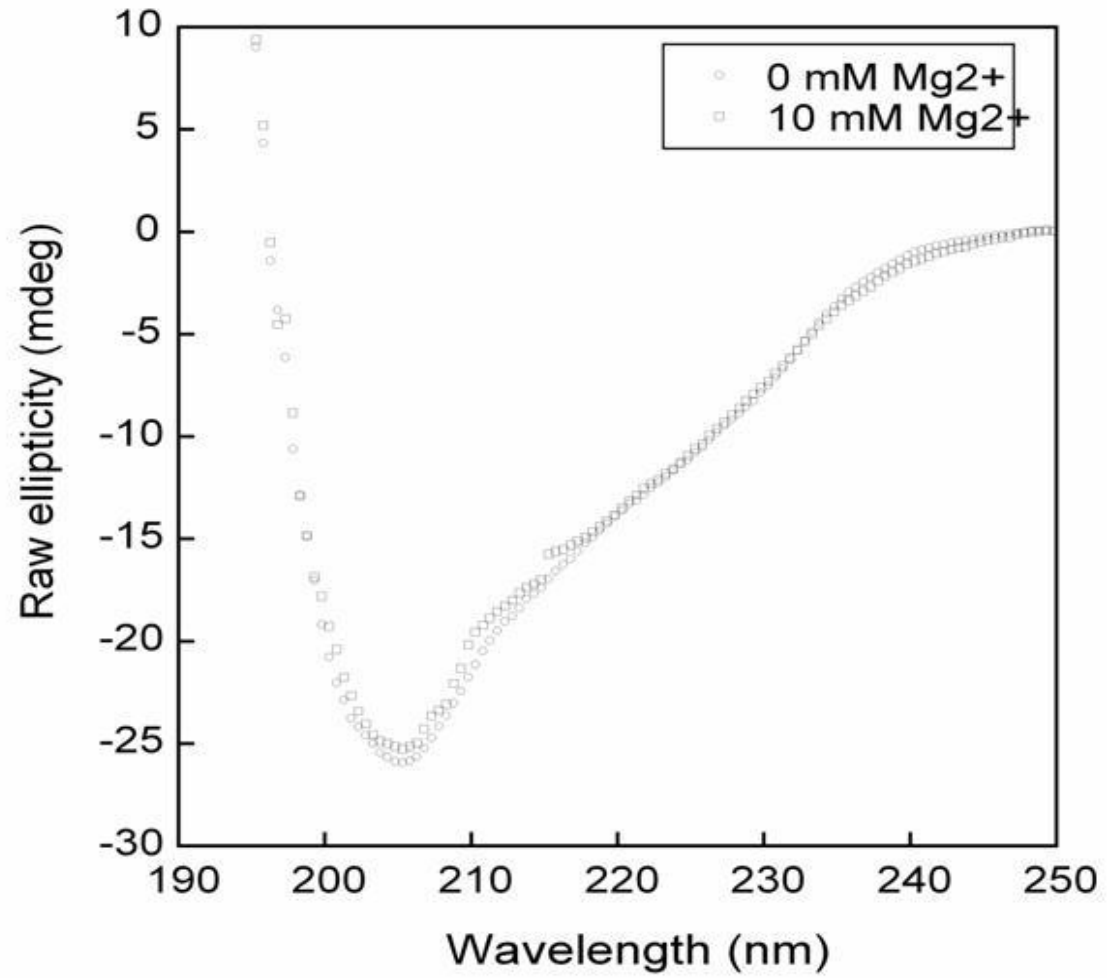


Fig. III.19. Far-UV CD spectra of the PhoQ sensor domain in the absence or presence of 10mM Mg^{2+}. Far-UV CD spectra of the PhoQ sensor domain in the absence (open circle) or presence (open square) of 10 mM Mg^{2+}.

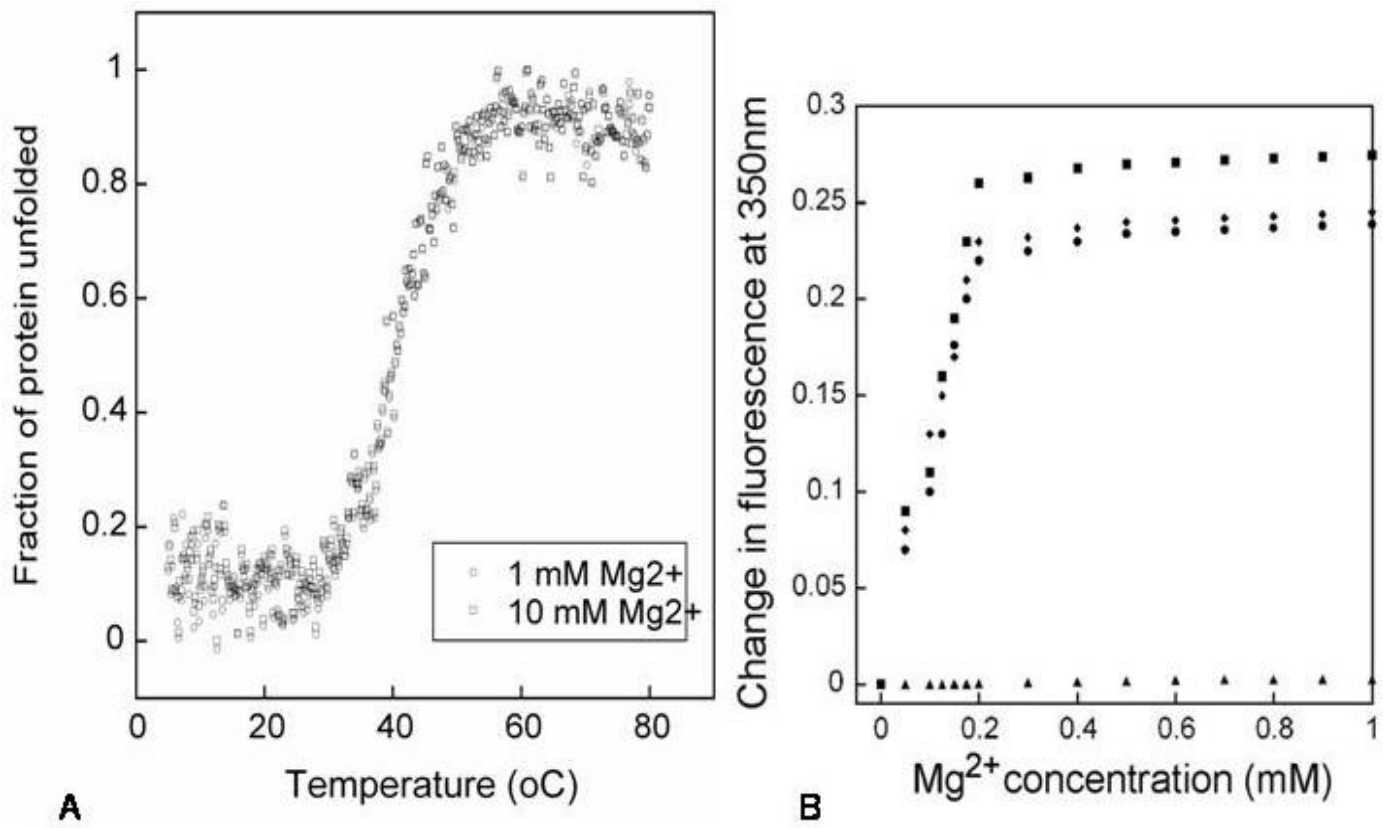


Fig. III.20. Binding of Mg^{2+} to the acidic cluster residues of PhoQ sensor domain.
(A) Thermal denaturation of the PhoQ NNNSANA acidic cluster mutant sensor domain followed by Far-UV CD in the presence of 1 mM (open circle) or 10 mM (open square) Mg^{2+}. (B) Binding of Mg^{2+} to the PhoQ sensor domain monitored by the absolute change in fluorescence at 350 nm at 20°C (closed rhombus), 30°C (closed square) and 37°C (closed circle). Mg^{2+} binding by the acidic cluster mutant, PhoQ NNNSANA, was monitored at 30°C (closed triangle).

To confirm that the acidic cluster residues of PhoQ are responsible for *in vivo* Mg^{2+} sensing in *E. tarda*, the *phoQ*$_i$ mutant was complemented with the *phoQ* gene carrying NNNSANA mutations, and the amounts of EseB (T3SS) and EvpC (T6SS) that were secreted, as well as expression level of *esrB*-LacZ, in the absence or presence of 10 mM Mg^{2+} were determined. In the presence of 10 mM Mg^{2+}, the *phoQ*$_i$ mutant complemented with the wild type *phoQ* showed a reduction in the secretion levels of both EseB and EvpC (Fig.III.21A). In contrast, the *phoQ*$_i$ mutant complemented with *phoQ* carrying NNNSANA mutations showed similar secretion levels of both EseB and EvpC and expression levels of *esrB*-LacZ in the absence or presence of 10 mM Mg^{2+}, suggesting that this cluster of acidic residues in PhoQ is essential for Mg^{2+} binding and concentration sensing for *E. tarda* (Fig. III.21B).

III.3.18. *E. tarda* can sense acidic pH and antimicrobial peptides

In addition to sensing Mg^{2+} concentration, the PhoQ sensor of *S. typhimurium* is also activated by acidic pH (Prost et al, 2007a) and antimicrobial peptides (Bader et al, 2005). To confirm if *E. tarda* can also sense acidic pH and antimicrobial peptides, the amounts of ECPs, as well as expression level of *ersB*-LacZ, in acidic culture medium (pH 5.5) and in the presence of an antimicrobial peptide (KR-20 or C-terminal 20 residues of cathelicidin LL-37) (Murakami et al, 2004) were determined. In acidic pH or the presence of antimicrobial peptide, *E. tarda* showed an increase in the secretion of ECPs from both T3SS and T6SS, as well as an increase in the expression level of *esrB*-LacZ (Fig. III.22 and Fig. III.23). The effect of antimicrobial peptide is more prominent than that of acidic pH and both effects are additive, suggesting that *E. tarda* can sense both acidic pH and antimicrobial peptides in addition to temperature and Mg^{2+} concentration. As residues from the acidic cluster of *Salmonella* PhoQ were previously shown to be involved in recognizing antimicrobial peptide (Bader et al, 2005), the effects of

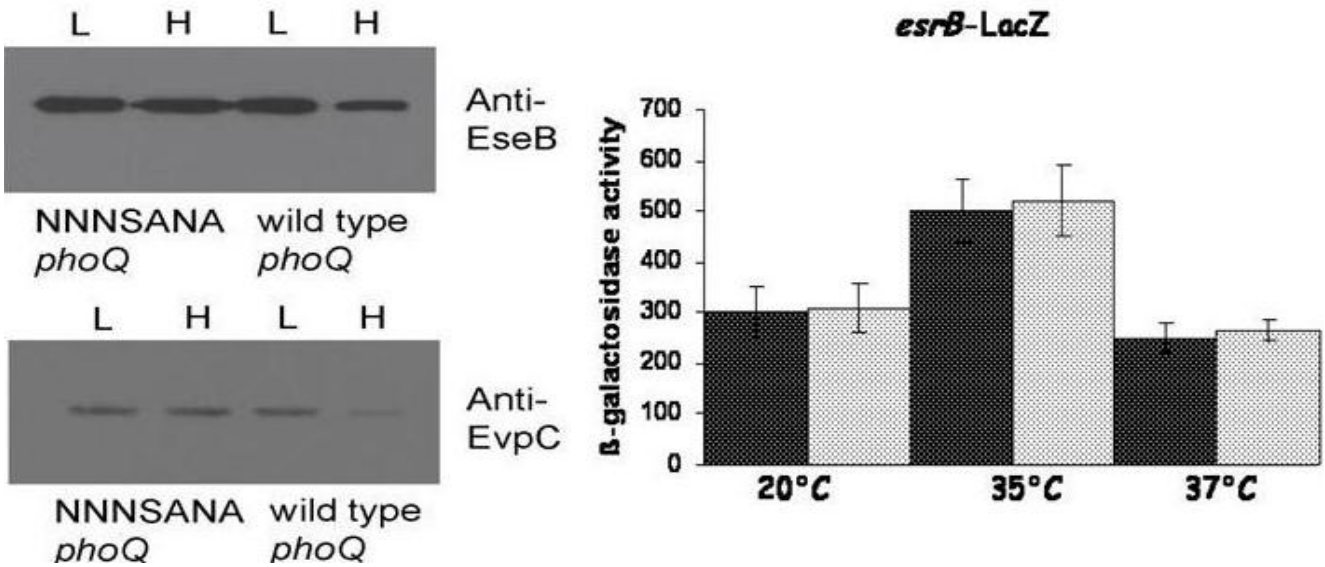


Fig. III.21. *E. tarda* acidic cluster mutant unable to sense Mg^{2+}. (A) Western blot analysis showing the secretion of EseB from T3SS (upper panel) and EvpC from T6SS (lower panel) by the *E. tarda phoQ*$_i$ mutant strain complemented with either *phoQ* NNNSANA or wild type *phoQ* in the absence (L) or presence (H) of 10 mM Mg^{2+} at 30°C. (B) The activity of the reporter gene *esrB*-LacZ at different temperatures in the presence of 1 mM (light grey bar) or 10 mM (dark grey bar) Mg^{2+} using the *E. tarda phoQ*$_i$ mutant complemented with *phoQ* NNNSANA. The values represent the mean ± SD from one representative experiment performed with triplicate samples. Equivalent results were obtained at least in triplicates.

antimicrobial peptide and acidic pH on the secretion of ECPs, as well as expression level of *esrB*-LacZ, were determined using *E. tarda phoQ*$_{i}$ mutant complemented with *phoQ* NNNSANA. Mutation of the acidic cluster residues activated secretion of ECPs and expression of *esrB*-LacZ, but addition of antimicrobial peptide had no further effect. In contrast, the secretion of ECPs and expression of *esrB*-LacZ in this *E. tarda* mutant are further activated by acidic pH, suggesting that the cluster of acidic residues in PhoQ is responsible for sensing antimicrobial peptide but not acidic pH (Fig. III.22 and Fig. III.23).

III.3.19.Effect of growth temperature on the TCP profile of *E. tarda*

To determine if there are any difference in the role of the PhoQ protein as a temperature sensor at high temperature (37^{o}C) and low temperature (20^{o}C), we performed two-dimensional gel electrophoresis (2DE) on the TCPs of the wild type as well as *phoQ$_{i}$* mutant strains of *E. tarda* at 3 different incubation temperatures of 20^{o}C, 35^{o}C and 37^{o}C. Growth of wild type *E. tarda* at 35^{o}C renders maximum proteins expression. A drastic decrease in the amount and number of proteins expressed was observed when the growth temperature was shifted to 20^{o}C or 37^{o}C, suggesting that a significant change in the protein expression pattern is required for the bacteria to adapt to or recover from adverse growth temperatures (Fig. III.24). Most interestingly, the profiles of TCPs expression are different at 20^{o}C in comparison to the profile at 37^{o}C, indicating two distinct physiological states of the bacteria.

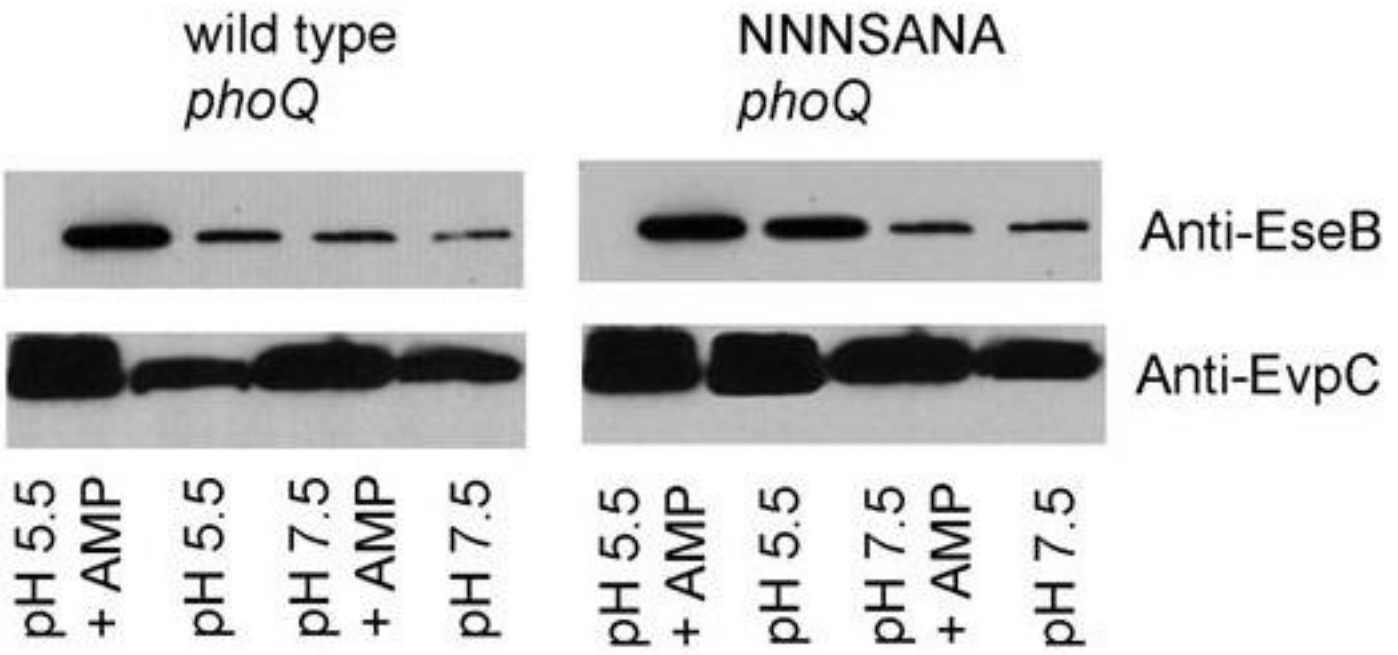


Fig. III.22. *E. tarda* PPD 130/91 responds to acidic pH and antimicrobial peptide. Western blot analysis showing the secretion of proteins from T3SS (EseB) and T6SS (EvpC) by *E. tarda phoQ*$_i$ mutant complemented with either *phoQ* NNNSANA or wild type *phoQ* grown in minimal medium at pH 5.5 (100 mM MES) or pH 7.5 (100 mM Tris-HCl) and in the absence or presence of 5 μg/ml of the antimicrobial peptide KR-20 (AMP).

In the *phoQ$_i$* mutant strain of *E. tarda*, the amount of TCPs is significantly reduced from that of the wild type strain at 35^{o}C (Fig. III.24). The result suggests the PhoP-PhoQ two-component system regulates expression of a large number of genes in *E. tarda*. When the growth temperature was shifted to 37^{o}C, *phoQ$_i$* showed similar expression profile of TCP to that at 35°C, suggesting that the *phoQ$_i$* mutant strain of *E. tarda* has lost the ability to detect the temperature change from 35^{o}C to 37^{o}C. The data also indicates that PhoQ is likely to be the prime sensor responsible for sensing temperature transition from 35°C to 37°C. In contrast, when the growth temperature was reduced to 20^{o}C, TCPs expression profile was moderately changed for the *phoQ$_i$* mutant, suggesting that in addition to PhoQ, there could be another temperature sensor which can detect temperature transition at 20 ^{o}C (Fig. III.24).

III.3.20 Effect of temperature on the morphology of *E. tarda*

To further elucidate the effect of temperature on the physiological state of *E. tarda*, the bacterial cells were grown at different temperature of 20^{o}C, 30^{o}C and 37^{o}C and their morphologies were observed under microscope. The growth temperature has a decisive effect on the shape of the bacterial cells. Cells grown at 20^{o}C acquired bacillus (rod shaped) phenotype, while at 37^{o}C attained coccus (sphere) phenotype. *E. tarda* grown at an optimum temperature at 30 ^{o}C show an intermediate cocco-bacillus phenotype (Fig. III.25).

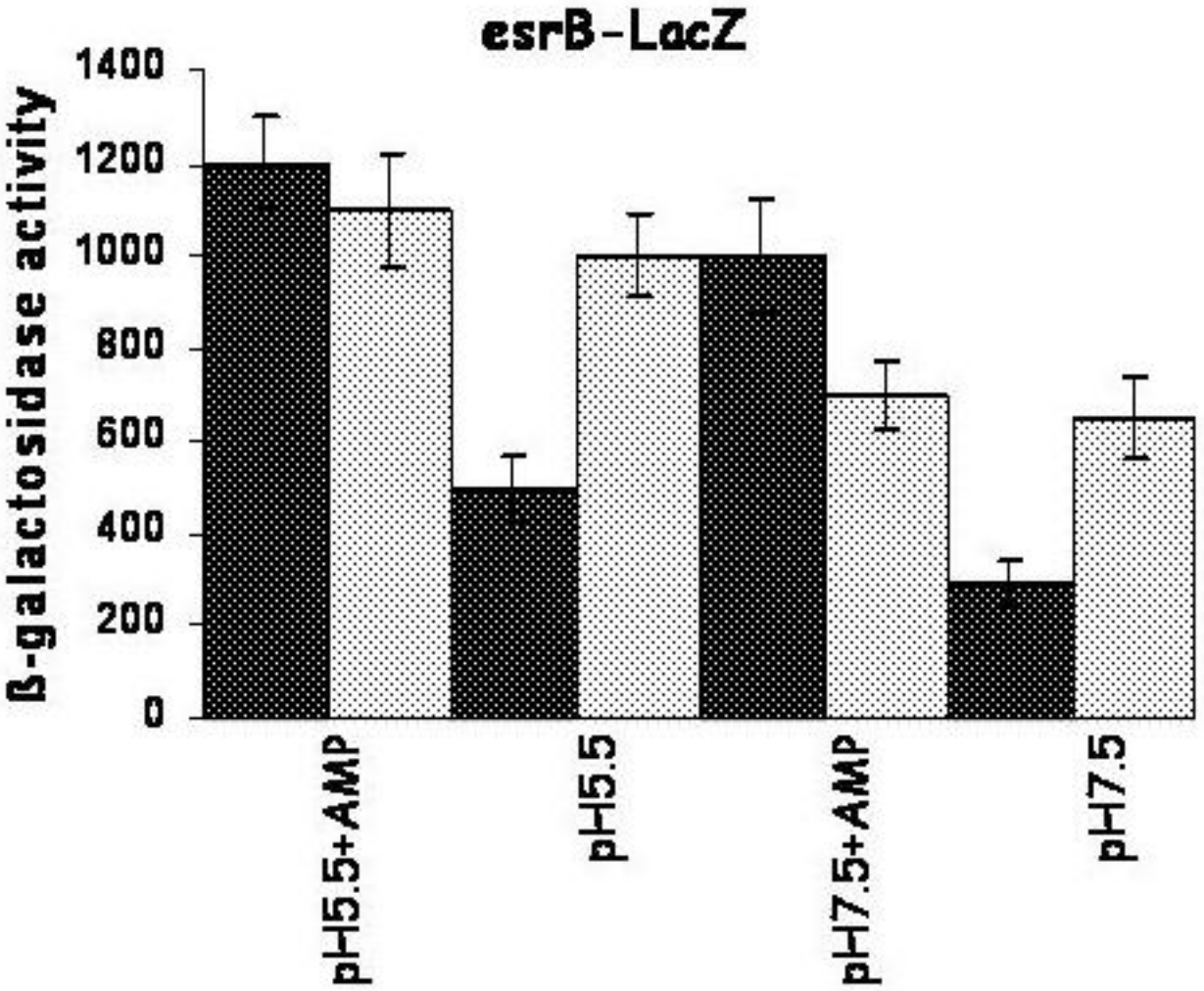


Fig. III.23. Acidic cluster residues in PhoQ is responsible for sensing anti-microbial peptide but not acidic pH. The activity of the reporter gene *esrB*-LacZ using *E. tarda phoQ*$_i$ mutant complemented with either *phoQ* NNNSANA (light grey bar) or wild type *phoQ* (dark grey bar) grown at pH 5.5 or pH 7.5 and in the absence or presence of 5 μg/ml of the antimicrobial peptide KR-20 (AMP). The values represent the mean ± SD from one representative experiment performed with triplicate samples. Equivalent results were obtained at least in triplicates.

III.4 Discussion

The EsrB regulator is encoded within the T3SS gene cluster and belongs to the response regulator of another two-component system called EsrA-EsrB. A functional EsrA-EsrB system is required to regulate the expression of EsrC, an AraC family transcriptional regulator that controls the expression of proteins encoded by T3SS and T6SS in *E. tarda* (Zheng et al, 2005b). Inspection of the promoter region of the *phoP-phoQ* genes revealed a putative PhoP box, with the sequence 5'-AGTTTA CCTAC CGTTGA-3', located upstream between nt -192 and -176; this sequences suggests that, in addition to regulating the levels of EsrB, PhoP may be involved in regulating the expression levels of both itself and PhoQ. Transcriptional autoregulation was also observed in the *phoPQ* operon of *S. typhimurium* (Soncini et al, 1995). The data herein demonstrated that the PhoP-PhoQ system senses temperature and Mg^{2+} concentration. In turn, the response regulator PhoP communicates the status of these environmental conditions to the EsrA-EsrB two-component system. This kind of "dual regulation" has also been observed in *S. typhimurium*, in which the SsrA-SsrB two-component system is regulated together with the two-component system OmpR-EnvZ, which responds to osmolarity, through the direct binding of OmpR to the promoter region of *ssrA* (Feng et al, 2003a; Lee et al, 2000a). Transcriptional regulation by a cascade of two-component systems allows pathogenic bacteria to express their virulence determinants in response to a broad spectrum of environmental cues. The PhoQ sensor in *E. tarda* can sense both temperature and

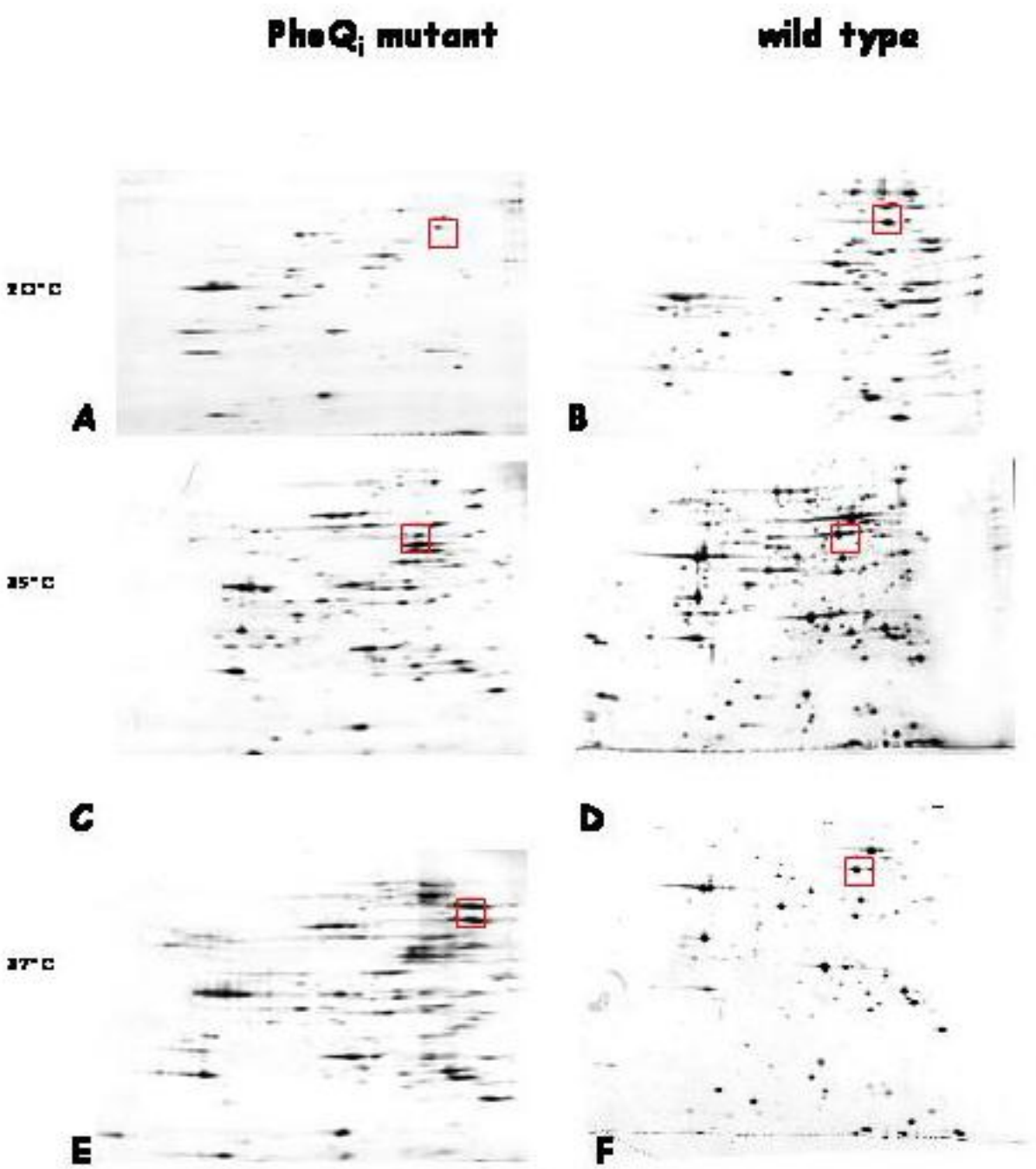


Fig. III.24. TCP profiles of wild type and *phoQ$_i$* mutant of *E. tarda* grown at different temperatures. Silver stained 2D PAGE showing the total cellular protein (TCP) profiles of wild type (right panels) and *phoQ$_i$* mutant (left panels) of *E. tarda* cultured in TSB at 20^{o}C, 35^{o}C and 37^{o}C. The TCP fractions were adjusted according to the relative amount of secreted protein from equal number of bacterial cells cultured at different temperatures. House keeping protein enolase in marked in red box.

Mg^{2+} concentration in an additive manner. We propose that it may integrate these signals with the signal detected by the EsrA-EsrB system to regulate the expression of proteins from T3SS and T6SS.

There was a significant decrease in the secondary structure (raw ellipticity from the CD spectra) of $PhoQ_s$ as the temperature increased, suggesting that temperature shift-induced conformational change occurs mainly on the level of the secondary structure rather than of the tertiary structure. $PhoQ_s$ likely has less secondary structure at 37°C than at 30°C but it still maintains its tertiary structure throughout this range of temperatures. The data also indicated that the conformations of $PhoQ_s$ at 20°C and 37°C could be different, even though both would have the ability to maintain $PhoQ_s$ in its inactive state. Sequence comparison with homologues from *S. typhimurium* and *E. coli* revealed that residues at positions 76, 77, 79, 120, 140 and 167 of $PhoQ_s$ are replaced with either Pro or Thr in *E. tarda*. The branched side chain of a Thr residue has been shown to generate instability at turn regions. Mutation of a Thr residue (Thr22) in the diverging β-turn of the drkN SH3 domain to a Gly residue results in a dramatic stabilization of the protein, with its T_m increased by 20°C (Mok et al, 2001). In contrast, a Pro residue is more rigid than other naturally occurring amino acids and could stabilize or destabilize a protein depending on its location (Prajapati et al, 2007). The use of a *phoQ*$_i$ mutant complemented with the wild type or mutant *phoQ* genes allowed those residues that are essential for thermal stability and temperature sensing to be delineated. Mutation of P79, P120 or T167 to their corresponding residues in the *E. coli* or S. typhimurium PhoQ sequence significantly stabilized the $PhoQ_s$ in *E. tarda*. The T167P mutant (T_m = 59.0°C) of *E. tarda* $PhoQ_s$ was thermally stable and its stability towards urea did not change at temperatures between 20°C and 37°C. These "loss-of-function" mutations improved the thermal stability of

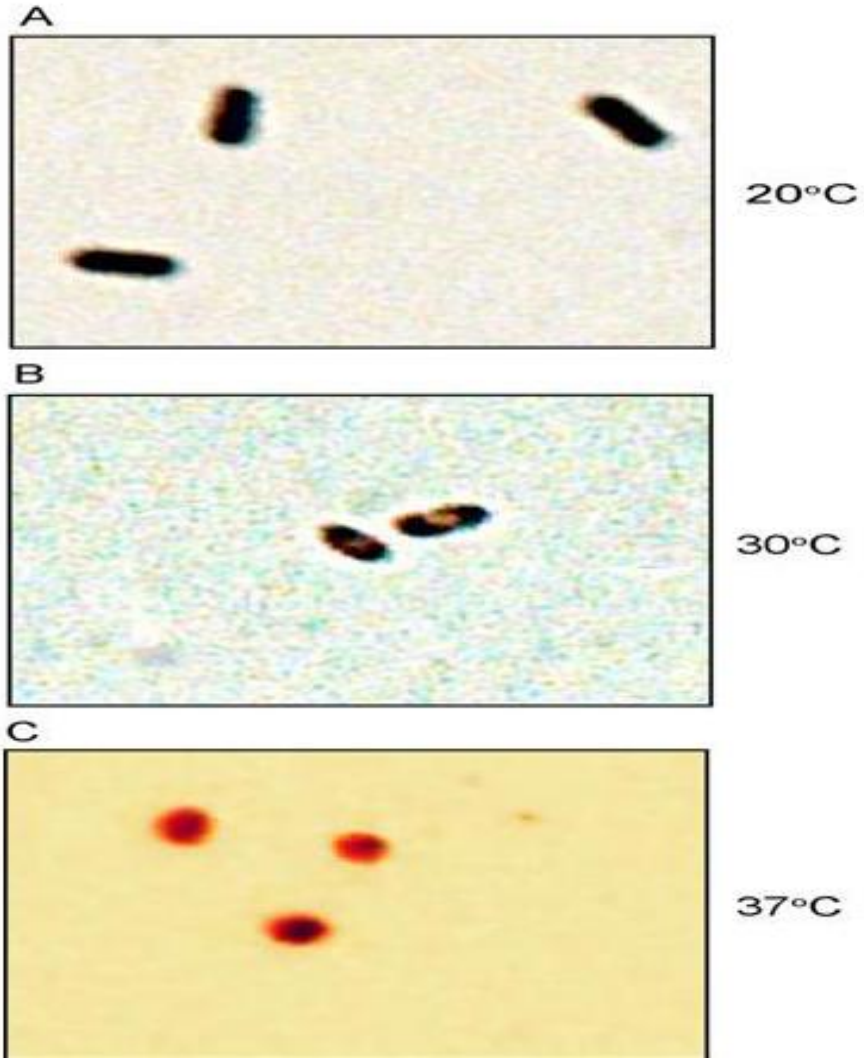


Fig. III.25. Morphologies of *E. tarda* bacterial cells cultured at different incubation temperatures. *E. tarda* bacterial cells grown in TSB assumed bacillus shape at 20^{o}C (A), coccobacillus shape at 30^{o}C (B) and coccus shape at 37^{o}C (C). The bacterial cells were gram stained and the images were obtained using Nikon Eclipse 90i objective: Plan Apo VC 60x/1.40 Oil at scale bar of 2 μM.

$PhoQ_s$ but rendered it unable to make the conformational changes necessary for the activation of PhoP. Consequently, no ECPs were detected at any of the tested temperatures, which was similar to results seen in the $phoQ_i$ mutant without any complementation. In contrast, the P77L mutant became "temperature-blind" at 20°C. This mutation prevented the secondary structure conformational change necessary to inhibit PhoQ activity at 20°C but not at 37°C. So far, no mutation that rendered the PhoQ sensor "temperature-blind" at 37°C or at both 20°C and 37°C has been identified. The observation that the P77L mutation only had an effect on temperature sensing at 20°C, but not at 37°C, also supported the notion that the conformations of the PhoQ sensor are different at these two temperatures.In this study, high Mg^{2+} concentration (10 mM) reduced the transcription of *phoP*, *phoQ* and *esrB* and thus reduced protein secretion from T3SS and T6SS in *E. tarda*. Mg^{2+} binding likely changed the tertiary structure, but not the secondary structure, of the PhoQ sensor. This explains the observation that Mg^{2+} binding by the PhoQ sensor was not affected by temperature at 20°C, 30°C or 37°C, which only resulted in changing the secondary structure of the protein, as well as the additive nature of the effects of Mg^{2+} and temperature on the PhoQ sensor, as the conformational changes caused by these factors could occur simultaneously and independently of each other. In *S. typhimurium*, Mg^{2+} is proposed to bind to the acidic patch "EDDDDAE" in the PhoQ sensor and form a metal bridge with negatively charged groups of the inner membrane (Cho et al, 2006). A similar, but distinct, acidic patch with the sequence "DDDSADA" is found within the PhoQ sensor domain of *E. tarda*. Our data confirmed that this cluster of acidic residues likely represents the only Mg^{2+} binding site on PhoQ because mutation of these residues to "NNNSANA" completely

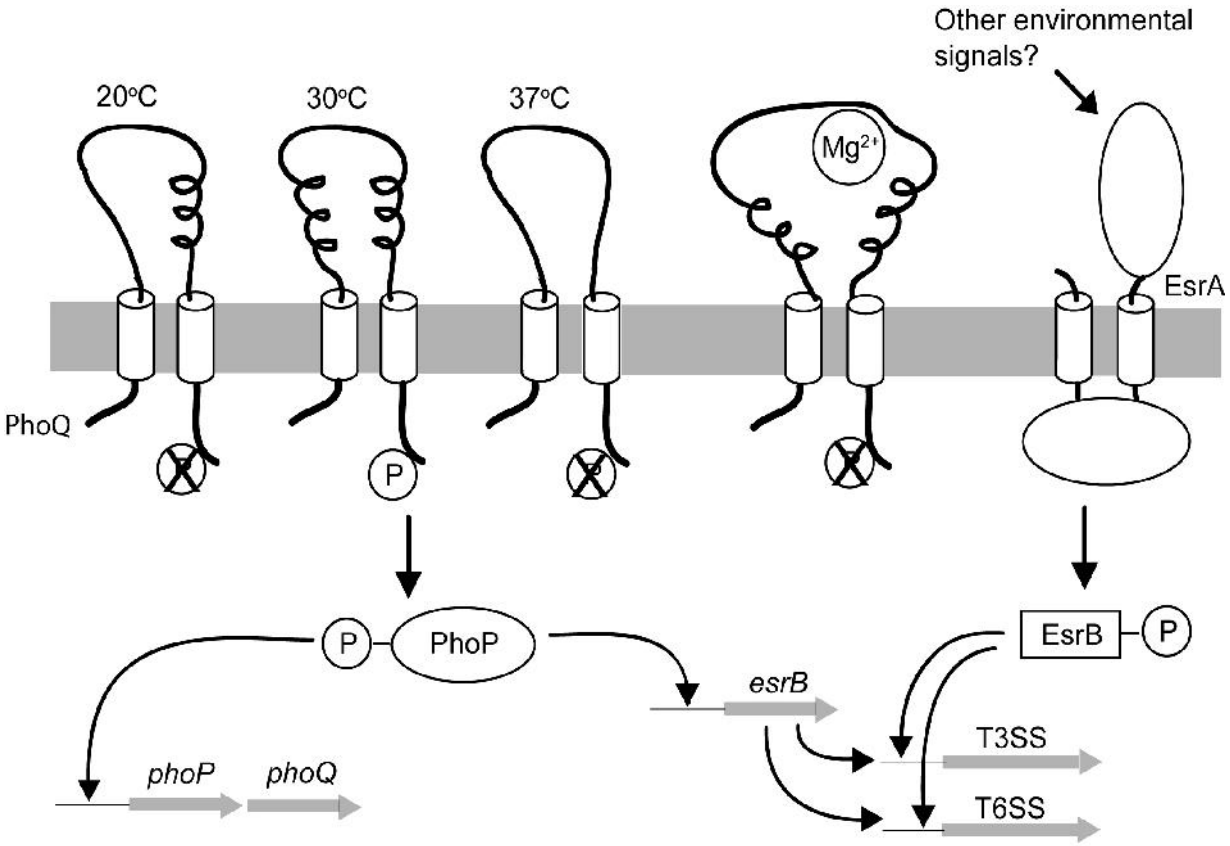


Fig. III.26. Model illustrating the temperature and Mg^{2+} regulation of T3SS and T6SS by the PhoP-PhoQ system.

abolished Mg^{2+} binding by the PhoQ sensor and because the *phoQ*$_i$ mutant complemented with this "NNNSANA" mutant is rendered "Mg^{2+}-blind". Mutations of this cluster of acidic residues did not affect the ability of PhoQ to activate PhoP and protein secretion from T3SS and T6SS or the ability of PhoQ to sense temperatures. Our study showed that Mg^{2+} bound to the PhoQ sensor domain of *E. tarda* at a relatively high affinity, with a K_d value of approximately 95 μM, comparable to the value obtained for the PhoQ sensor domain of *P. aeruginosa* (K_d = 37 μM for Ca^{2+}; K_d = 207 μM for Mg^{2+}) (Prost et al, 2008) but distinct from that of the PhoQ of *S. typhimurium* (K_d = 250 μM for Ca^{2+}; K_d = 7 μM for Mg^{2+}) (García Véscovi et al, 1997). Interestingly, the *P. aeruginosa* PhoQ lacks this acidic patch of residues, and, unlike the *E. coli* protein, the *P. aeruginosa* PhoQ sensor domain undergoes changes in its CD and fluorescence spectra in response to divalent cations (Lesley & Waldburger, 2001). These data suggested that the PhoQ sensors of *E. tarda*, *S. typhimurium* and *P. aeruginosa* may have very different mechanisms of signal detection. The high affinity binding of Mg^{2+} also increased the stability of the PhoQ sensor slightly, as monitored by both CD and fluorescence spectra. This increased stability is likely due to the neutralization of electrostatic repulsion among residues within the acidic patch. Unlike the situation with the T167P mutation, this slight increase in stability due to Mg^{2+} binding is not sufficient to disrupt temperature detection by PhoQ.

E. tarda infects many different fish species, such as blue gourami fish and channel catfish. The body temperature of catfish fluctuates with and approximates the surrounding water temperature. Their active metabolic rate increases as a hyperbolic function of temperature, reaching a peak at around 28°C-30°C. Reduced feeding by the fish should occur at the higher temperature of 35°C, with none at 36°C-38°C. Alternatively, there should also be reduced

feeding by the fish at the lower temperature of 15^{o}C and no feeding at 8^{o}C-10^{o}C (Hargreaves & Tomasso Jr., 2004). This growth temperature profile of fish is in agreement with our findings on the temperature dependence of protein secretion from the *E. tarda* T3SS and T6SS, suggesting that the PhoP-PhoQ system of *E. tarda* is only activated at the optimum growth temperature of the host to ensure the highest level of virulence and the survival of the bacteria. These findings also agree with the observation that outbreaks of acute *E. tarda* infection are mostly found in channel catfish culture systems when the temperature rises due to overcrowding (Mohanty & Sahoo, 2007). On the other hand, Mg^{2+} concentration detection by the PhoP-PhoQ system seems to provide *E. tarda* with cues that it located inside the host body. At a physiological level of around 1-2 mM, the Mg^{2+} concentration inside the host body is generally lower than that of the external environment (Bader et al, 2005). Direct measurement of Mg^{2+} within the *Salmonella*-containing vacuole using nanosensor particles showed that, during the initial period of *phoP* activation, the concentration of the divalent cation was rapidly regulated and stabilized at approximately 1 mM (Martin-Orozco et al, 2006). In addition to temperature and Mg^{2+} concentration, *E. tarda* can also sense both acidic pH and the presence of antimicrobial peptides, which are characteristics of environment inside the phagosome of macrophage.

In conclusion, we cloned the PhoP-PhoQ two-component system of *E. tarda* and confirmed that the PhoQ sensor domain senses changes in temperature through conformational changes in its thermally unstable secondary structures. The PhoQ sensor can also simultaneously detect changes in Mg^{2+} concentrations through the direct binding of Mg^{2+} to a cluster of acidic residues, which likely results in a change in the tertiary structure of the protein. Both the temperature and Mg^{2+} signals are integrated with the signal detected by another two-

component system, EsrA-EsrB, through the direct activation of the transcription of the EsrB response regulator by PhoP. The data herein provides a basic understanding of the mechanism underlying the temperature and Mg^{2+} sensing abilities in bacteria and could aid future structural studies of the system as well as control of the pathogen.

Chapter IV. Crosstalk between phosphate and iron mediated regulation of Type III and Type VI secretion system in *E. tarda*

The results of this chapter are included in the following manuscript:

Chakraborty S, Sivaraman J, Leung KY, Mok YK: Crosstalk between Phosphate and Iron mediated Regulation of Type III and Type VI secretion system in *E. tarda*
Manuscript in preparation**.**

Abstract

The pathogenic bacterium *Edwardsiella tarda* employs both type III and VI secretion systems (T3SS and T6SS) to inject multiple effectors into eukaryotic cells. The PhoB-PhoR two-component system is commonly used by pathogenic bacteria to sense low phosphate concentration in the environment and in turn regulates the high affinity phosphate transporter *pstSCAB-phoU*. On the other hand, Fur is an iron sensor which is triggered by a high concentration of iron in the surrounding media. Here, we show that PhoB/PhoR in *E. tarda* detect changes in phosphate concentration, as well as positively regulates the T3SS and T6SS through direct activation of *esrC*. Protein expression and secretion is activated in absence of phosphate and is suppressed in presence of 20mM phosphate concentration. The Fur protein acts as an iron sensor which negatively regulateT3SS and T6SS at high concentration of iron through the modulation of *esrC*. Iron and phosphate act in an additive manner in which iron can also suppress the activities of *phoB/phoR* and *pstSCAB-phoU* operons. On the other hand, phosphate enhances the transcriptional activity of *fur*. Here, we show a negative cross-talk that exists between the high affinity phosphate transport specific regulon pstSCAB-phoU and the iron sensor Fur.

IV.1 Introduction

Iron homeostasis is essential for the maintenance of bacterial population. Since most life forms contain iron in their cells, regulating homeostasis of this metal is vital for normal cell functioning. Iron also serves as a cofactor for many enzymes. Iron is absolutely necessary for the survival since it plays an important role in the TCA cycle and electron transport chain. However, excess amount of free iron can also produce reactive oxygen and nitrogen species that can damage the cellular components (Friedman & O'Brian, 2004) . Vertebrates sequester iron and other nutrients to prevent the outgrowth of pathogens and this kind of defense mechanism is known as nutritional immunity. The neutral pH and the aerobic environment ensure that the iron is available in the insoluble form and cannot be consumed by the bacteria. Moreover, there are chelators which are iron binding proteins (transferrins, lactoferrins and ferritins) that can bind to iron and reduces the availability of free iron to the invading bacteria. Hence recognition of iron depletion has become a marker of vertebrate tissue for bacteria (Skaar, 2010). In order to evade nutritional immunity by the host, bacteria have evolved three mechanisms. In siderophore based system, siderophore have better iron binding capacity with an association constant of 10^{50} as compared to transferrin ($Ka=10^{36}$) and these siderophore-iron complexes are internalized as a nutrient source. Siderophores from the outer membrane are translocated through an energy dependent process into the cytoplasm. Transport of ferrous iron and ferric iron bound to siderophores that takes place through different transport systems such as the substrate binding protein (FhuD in *E. coli*) and integral membrane proteins (FhuB in *E. coli*) which belong to the ABC transporters (Köster, 2001)**.** Under iron rich conditions, the synthesis of siderophores are normally repressed. Overproduction of siderophores are seen in *E. coli* and *Salmonella typhimurium* in the *fur* mutant background (Hantke, 2001). In heme

acquisition system, heme scavengers homophores remove heme from the host and through the recognition of homophores by the surface receptors of the bacteria. In the third transferrin receptor system, some bacteria can directly recognize transferrin by means of the receptors where the iron is removed from the host and integrated to the bacterial cytoplasm. A second layer of defense is also employed by the host in which host lipocalin binds to enterobactin, which is the prime siderophore of many enteric bacteria. In response to that, bacteria have also evolved stealth siderophore that does not bind to lipocalin (Skaar, 2010)**.** Iron dependent repressor Fur (Ferric uptake regulator) is a dimeric metallo-protein where the N-terminal region contains a DNA recognition domain and the C-terminal region is involved in dimerization and metal binding, such as Mn(II), Ni(II), Cd(II), Zn(II) and Fe(II) (Coy & Neilands, 1991). Fur binds to its specific promoters containing the Fur box having a consensus sequence of 5/-GATAACGCAATGATTGATA-3/ and represses the expression of targeted genes in presence of iron. In the absence of iron, these genes are transcribed as the repression activity is inhibited. In Gram positive bacteria, an additional iron repressor is present which belongs to the DtxR family (Skaar, 2010). Fur also negatively auto-regulates its own transcription in the presence of high iron concentration. Cys-92, Cys-95 and Glu-112 are essential for its functioning as mutations of these residuesabolished its activity. Two zinc ions are present in each monomer of Fur. Zinc can be removed by EDTA treatment which rendered Fur unable to bind to DNA strands. Addition of metals like Zinc (Zn^{2+}) and Ferric (Fe^{3+}) restored the DNA binding capacity (Sheikh & Taylor, 2009)**.** Microarray analysis revealed several candidates which are repressed, induced or remain indifferent to iron concentrations. Majority of the genes involved in iron acquisition were down regulated. 14 novel genes are negatively regulated by Fur which play no role in iron transport. Iron containing form of super

oxide dismutase (encoded by *sodB*) that is responsible for protecting the cells during low iron conditions is repressed by Fur. Whereas *sodA* which encodes the manganese containing form is activated. This phenomenon is seen in several species such as *Salmonella enterica serover Typhimurium* (Bjarnason et al, 2003), *E. coli* (Niederhoffer et al, 1990) and *Vibrio cholerae* (Mey et al, 2005a). Fur is also known to repress Bfr (ferritin-like protein) and induce Bfd (ferrodoxin). Bfd is up regulated and Bfr is repressed under low iron conditions in *Salmonella* (Bjarnason et al, 2003) and *E. coli* (Quail et al, 1996; Stojiljkovic et al, 1994). However, in *Vibrio cholerae* both the Bfr and Bfd were up regulated in low iron conditions (Davis et al, 2005). Apart from microarray analysis, various other tools have been employed to identify the role of Fur apart from iron homeostasis. Fur titration assay (FURTA) (Stojiljkovic et al, 1994) used a cosmid library to fish out iron-regulated promoters. Another method known as SELEX (Systemic evolution of ligand s by exponential enrichment) identified 16 novel genes regulated by Fur in *P. aeruginosa* (Ochsner & Vasil, 1996). A combination of two-dimensional electrophoresis followed by reverse genetics have identified 10 iron regulated proteins of *M. tuberculosis*. (Wong et al, 1999). Various other roles of Fur has been established which are not related to iron metabolism such as defense against reactive oxygen species (Niederhoffer et al, 1990; Tardat & Touati, 1993); acid shock response (Hall & Foster, 1996); chemotaxis (Karjalainen et al, 1991); metabolic pathways (Stojiljkovic et al, 1994), swarming (McCarter & Silverman, 1989) and virulence factors (Litwin & Calderwood, 1993) (Escolar et al, 1999). Some of the genes, e.g. *tonB1* / *hutW* in *Vibrio cholerae* are highly induced by Fur which contain more than one Fur box in their promoter region increasing the magnitude of recognition by Fur (Occhino et al, 1998; Wyckoff et al, 2004). Fur also plays an important role in virulence in many bacteria such as *Bacillus cereus* (Harvie et al, 2005), *Staphylococcus*

aureus (Horsburgh et al, 2001), *Actinobacillus pleuropneumoniae* (Jacobsen et al, 2005), *Listeria monocytogenes* (Garcia et al, 2004), *Campylobacter jejuni* (Palyada et al, 2004) and Vibrio cholerae (Chiang et al, 1995) (Mey et al, 2005b). In *E. tarda* EIB202, transcription of evpP is dependent on both EsrB and iron and the transcriptional activity of *evpP* was increased in iron rich medium. Flow cytometry assay showed that FuR box is present upstream of *evpP*. EvpP is also important for virulence since a knock out mutant of *evpP* showed a marked attenuation in LD_{50} with decreased internalization in EPC cells in comparison to the wild type bacteria. EvpP also play a role in hemolytic activity as well as adherence to the mucous membrane in Japanese flounder. However, EvpP is not involved in biofilm formation and production of chondriotinase (Wang et al, 2009b).

Apart from iron, phosphate is another fundamental nutrient for bacterial cells. *E. coli* used 3 different kinds of phosphates, namely inorganic phosphate, organophosphate and phosphonates, of which inorganic phosphate is highly preferable. Phosphorus is essential for cellular functioning such as regulation of protein activities, source of ATP, synthesis of DNA, RNA and amino acids. Bacteria have developed ways to assimilate phosphate when it is present in abundance or in case of scarcity. When the Pi source is in excess, phosphate is taken up by phosphate inorganic transporter (Pit) system. High affinity phosphate transporter pstSCAB is triggered when the concentration of extra-cellular phosphate is less than 4μM. They belong to the ATP-binding cassette transporter of the pho regulon. The last gene of this operon, *phoU*, encodes PhoU protein in which contains two PhoU homologues are also found in *Thermotoga maritima*. Pi signaling involves activation, deactivation and inhibition. Activation happens during Pi limiting condition, which requires auto-phosphorylation of PhoR,

transfer of phosphoryl group to PhoB, and binding of phosphor-PhoB to its targeted genes for transcriptional activation. Deactivation occurs during Pi excess condition, where PhoR and the entire pst operon are required for the dephosphorylation of PhoB. In Inhibition, the phosphorylation of PhoB is abrogated through formation of an inhibitory comples with proteins belonging to the pst operon (Haldimann et al, 1998). Phopshorylated PhoB binds to a particular DNA sequence 5/-C**TGTCA**TA(A/T)A(T/A)C**TGTCA**(C/T)-3/ which is known as the pho box. PhoB binding to the *pstS* promoter induces a possible bending (Makino et al, 1996)**.** PstS is a cell surface receptor for inorganic phosphate. PstC and PstA are integral membrane proteins, and PstB is a permease. The last gene of the operon codes for PhoU which has no role in transport but might act as a suppressor of pho regulon. PhoR has dual forms as an activator $PhoR^A$ or a repressor $PhoR^R$. PhoR is an inner membrane histidine complex, which upon induction transfers the phosphoryl group to an aspartic acid residue on PhoB, when the phosphate concentration is below 4 μM. PhoR consists of an H box (His-159 to Leu-176; His165 putative phosphorylation site), an N motif (Asn-267 to Tyr-285), a D/F (Ile-307 to Asp-334) and finally a G (Gly-343 to Val-362) box. PhoP gene belongs to the OmpR family of proteins where Asp-6 and Asp-9 are the putative phosphorylation sites. The carboxyl terminal region consists of residues from 190 to 201 which is the DNA binding motif. Under Pi repressing condition, Pi bound PstS forms a complex with PstABC which maintains PhoR in $PhoR^R$ form. Pi –independent controls are also seen where CreC and acetyl phosphate can phosphorylate PhoB (Wanner, 1993). Nearly 400 genes in *E. coli* respond to changes in phosphate concentration, which comprises 10% of its own genome. PstB and PhoU are required for the dephosphorylation of phosphor-PhoB, while PhoU may act as a chaperon to the PhoB/PhoR complex. Bacteria have adapted novel ways to survive in limitation conditions.

The shape of the bacterial cells can be changed to increase the surface to volume ratio. The cell wall component can be modified from phosphate rich techoic acid to phosphate free teichuronic acid. Other than phosphate regulation, the pst regulon are also important for virulence. In *E. coli*, *pst* mutants become avirulent in pig infection model. Pst mutations reduced the resistance of bacteria against anti-microbial peptides, serum killing and acidity. Transposon mutants of *pst* in *E. tarda* reduced the capacity to multiply in the phagocytes and these mutants are unable to secreted any Type III and Type VI proteins. Iso-genic mutants in PhoB and PhoR showed decreased colonization in rabbit in comparison to the wild type. In *Vibrio*, much virulence related genes are suppressed in *phoB* mutant strain. PhoB/PhoR may be involved in the circuit that control acid stress response. Bacteria develop biofilms to protect themselves from environmental stress. Pst mutants have been shown to hinder biofilm formation. In *E. coli*, *pst* mutantions also affect quorum sensing which suggest that Pst genes can work through a complicated cascade and regulate the formation of biofilms and resistance against host defense that target cell surfaces (Lamarche et al, 2008)**.**

Genome wide prediction of PhoB regulated promoters is performed by reporter gene fusion assays using *in silico* prediction and microarray data in gram negative ά-proteobacterium *Sinorhyzobium meliloti*. PhoB binding motif was found in 96 pho regulon members, of which 34 were directly related to PhoB in a Pi dependent manner. Out of the 34, 19 genes possessed the putative PhoB binding box in their respective promoter regions (Yuan et al, 2006). Genes regulated by pho regulon mainly comprised of alkaline phosphatases (AP), phosphate specific transport (pst) and two-component regulatory system (PhoB/PhoR). Pho regulon is also controlled by central carbon pathways where CreC (PhoM) act as an alternative sensor kinase,

suggesting interaction between PhoB/R and CreC. The expression creC was high in absence of Pho but low in absence of PhoB. In the initial phase of phosphate starvation, CreC first interacts with PhoB and then followed by PhoB- PhoR. PhoU also responds to phosphate starvation and its level is decreased in the absence of PhoB. However, FRET confirmed that PhoU does not interact directly with PhoB and PhoR. Thus, it is postulated that PhoU may interact with proteins in the Pst system which in turn can interact with PhoB/PhoR (Baek et al, 2007). In *E. coli*, *phoU* mutant has derepressed alkaline phosphatase activity in comparison to the wild type bacteria under high phosphate condition. PhoU mutant showed decreased colonization in murine urinary tract. No growth defect is observed in this mutant (Buckles et al, 2006). The exact mode of action of PhoU remains to be determined.

IV.2 Materials and Methods

IV.2.1 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table II.1 and Table IV.2. When required, media were supplemented with Amp (100 μg/ml), Cm (30 μg/ml) or Tc (10 μg/ml).

IV.2.2 Cloning of the PhoB-PhoR two-component system in *E. tarda*

Bacterial genomic DNA was extracted using the Wizard genomic DNA purification kit (Promega, Madison, WI). Degenerate primers were designed to fish out the complete genome sequences of *phoB* and *phoR* along with flanking regions from related bacteria such as *Edwardsiella tarda EIB202* (Wang et al, 2009a); *Yersinia pestis KIM* (Deng et al, 2002a); *Salmonella typhimurium LT2* (McClelland et al, 2001b) and *Escherichia coli CFT073* (Welch et al, 2002a). PCR amplification (2 min at 94°C; 30 cycles each of 10 s at 94°C, 30 s at 56°C

and 1 min at 72°C; and a final extension of 5 min at 72°C) was carried out using the Advantage 2 polymerase mix (Clontech, Mountain View, CA) with two pairs of degenerate primers, *phoB*deg and *phoR*deg (Table IV.1). The PCR products were cloned with the pGEM-T Easy vector system (Promega) and transformed into *E. coli* DH5α cells. The cloned fragments were sequenced using the PRISM^{TM} 3100 automated DNA sequencer with the ABI Prism Big Dye termination cycle sequence kit (Applied Biosystems, Foster City, CA). The complete sequences of *phoB* and *phoR*, including flanking sequences comprising the 1,360 bps upstream of *phoB* and 1,400 bps downstream of *phoR*, were obtained by this method.

IV.2.3 LacZ reporter gene system

For the construction of the *ersB* LacZ and *esrC* LacZ reporter gene systems, the primer pair, pRW*esrb* and pRW*esrc* derived from *E. tarda* PPD130/91 genomic DNA, was used to amplify the putative promoter region of *esrB* and *esrC*. The PCR product was digested with restriction enzymes and then ligated with the pRW50 plasmid (Lodge et al, 1992). The ligation mixture was introduced into either the wild type or mutant *E. tarda* by electroporation. The transformants were screened for resistance to both colistin (12.5μg/ml) and tetracycline (10 μg/ml). Similar procedures were followed to generate the *phoB* and *fur* LacZ reporter gene systems using the primers pRW*phoB* and pRW*fur*, respectively. For the construction of pVIK*phoU*, the *phoU* internal fragment was amplified with the primer pVIK*phoU* (Table IV.1) from *E. tarda* PPD130/91 genomic DNA. The PCR product was digested and then ligated into the same sites of pVIK112 . The resulting plasmids were transformed into S17-1 λpir. The plasmids were then transferred from S17-1 λpir into *E. tarda* by conjugation and the LacZ transcriptional or translational strains were selected for kanamycin and colistin resistance. For

the β-galactosidase assays, *E. tarda* cells were grown in DMEM overnight at 30°C. Phosphate and iron were added in the form of KH_2PO_4 and $FeSO_4$ as indicated. The overnight culture (5%) was inoculated into fresh medium and grown at the same conditions until the cell density reached 0.5, as measured by optical density at 600 nm (OD_{600}). Cells were permeabilized as described by Miller, 1972 and in Chapter III.

IV.2.4 Generation of *phoU*, *phoB* and *fur* mutants and complementation experiments

Insertional mutants of *phoU* (*phoU*$_i$) in *E. tarda* was constructed with the suicide plasmid pRE112 (Edwards et al, 1998). For the construction of *phoU*$_i$, an internal fragment of *phoU* was amplified from *E. tarda* genomic DNA with the primer *phoU*$_{mut}$-for and *phoU*$_{mut}$-rev which contains a *Kpn* I restriction enzyme site. The PCR product was digested by restriction enzymes and ligated into the pRE112 plasmid, and the resulting plasmid was transformed into *E. coli* MC1061 λpir. After sequencing, the recombinant plasmid was then transformed into *E. coli* SM10 λpir. These transformants were used to conjugate with wild type *E. tarda* to obtain defined mutants by selecting colonies resistant to both chloramphenicol (30 μg/ml) and colistin (12.5 μg/ml). The insertion of the plasmid into chromosomal DNA was confirmed by sequence analysis. Complementation of the *phoU*$_i$, mutant was performed with a pACYC184-based system. To obtain *phoU*$_i$ + *phoU*, the complete *phoU* gene was prepared by PCR using the primer pair, *phoU*$_{full}$-for and *phoU*$_{full}$-rev (Table IV.1), from *E. tarda* PPD130/91 genomic DNA. The PCR product was digested with restriction enzymes and ligated into the digested pACYC184 plasmid. The obtained ligation mixture was transformed into *E. coli* DH5α, and then the plasmid DNA was extracted and transformed into competent cells of *E. tarda phoU*$_i$ by electroporation.

Overlap extension PCR (Steffan N. Hoa, 1989) was used to generate in-frame deletion mutants of *phoB* (*ΔphoB*) and *fur* (*Δfur*). For the construction of Δ*phoB*, two PCR fragments were generated from *E. tarda* genomic DNA with the primer pairs of phoBmut-for / $phoB_{mut}$-irev and $phoB_{mut}$-rev / $phoB_{mut}$-ifor (Table IV.1). The resulting products generated an 820-bp fragment containing the upstream region of *phoB* and 850-bp fragment containing the downstream region of the *phoB*, respectively. An 18-bp overlap in the sequences permitted amplification of a 1670-bp product during a second PCR with primers of $phoB_{mut}$-for and $phoB_{mut}$rev using the first two PCR products as the template. The primers introduced an EcoRI and a HindIII restriction sites and the second PCR created a truncation from amino acid 15 to 230 of PhoB. The PCR product was cloned into pGEM-T Easy vector, and DNA sequencing was performed to confirm that the construct was correct. The Δ *phoB* fragment was excised with the same restriction sites and ligated into EcoRI and HindIII cut suicide vector pRE112 (Cm^r) (Edwards, 1998). The resulting plasmid was then transformed into MC1061λpir. After sequencing, the recombinant plasmid was then transformed into *E. coli* SM10 λpir. The single cross-over mutants were obtained by conjugal transfer into *E. tarda* PPD130/91. Double cross-over mutants were obtained by plating onto 10% sucrose TSA plates. Deletion mutants were confirmed by PCR and sequencing. Fur deletion mutant (*Δfur*) was constructed following the same protocol. The primers used for the construction are fur_{mut}-for, fur_{mut}-irev, fur_{mut}-rev and fur_{mut}-ifor. These primers introduced Kpn-I as the restriction enzyme cloning site. This approach created a deletion from amino acids 15 to 100 of Fur.

Table IV.1. Oligonucleotides used in this study

Name	Sequence (5' to 3')
pRW*esrb*-for	ATGAATTC GTGGCGGTGGATTATGACGAT
pRW*esrb*-rev	ATAAGCTT CTTACTCGATCTGGGTCTGCC
pRW*esrc*-for	ATGAATTC TATGATCATCACGCTGCAGGA
pRW*esrc*-rev	ATAAGCTT CCGCTGTACATCAGGTGCTG
pRW*phoB*-for	CG GAATTC AATGTGTCCCAGGGCGATATAG
pRW*phoB*-rev	CG AAGCTT GGTCATACTGACTTCCCGGTT
pRW*fur*-for	CG GAATTCCCGGATACCACTTTGAAGCCT
pRW*fur*-rev	CG AAGCTT GTCAAACTGGTTGAGCACGCGG
pRW*pst*-for	ATGAATTC TGTGAGCATAGCCATGAGATG
pRW*pst*-rev	AAGCTT TTTCTGATAGGAGTCTGCCCA
pVIK*phoU*-for	CG GAATTC AATCTGAATTTAAACAAGCACA
pVIK*phoU*-rev	GG GGTACC CTCACAGATATTCTGGCAGCGATCG
$phoU_{mut}$-for	ATGGTACCCAGTTTAATGCCGAGCTT
$phoU_{mut}$-rev	ATGGTACCGATAGAGCGGGCGCAGAAC
$phoU_{full}$-for	ATAGTACTATGGATAATCTGAATTTAAAC
$phoU_{full}$-rev	ATGAATTCTTATGCCTCGTCCACCCGG
$phoB_{mut}$-for	ATAAGCTTCGCGGGCATAGCTGGGGGG
$phoB_{mut}$-irev	ACGGGCGGAGAAACGGTA CTGACTTCCCGGTTAATTGTCAT
$phoB_{mut}$-rev	ATGAATTCCGTATGGTTGATCGCGTTATA
$phoB_{mut}$-ifor	TACCGTTTCTCCGCCCGT
fur_{mut}-for	GG GGTACC AGGAGATCGACTTCTCCGGTAA
fur_{mut}-irev	TTAGGCCTTTTCGTCGTGCA ATGTCGATCAGCTTCTTGTACAA
fur_{mut}-rev	GG GGTACC GCGTACCGATGAGATGATGGAG
fur_{mut}-ifor	TGCACGACGAAAAGGCCTAAGCGGCC

Rt-*3537S*$_{1/2}$ for	TAGCGGGAAAGGGTAGACGATA
Rt-*3537S*$_{1/2}$ rev	ACGGTGTTAGCAATGATCTGC
Rt-*SC*$_{1/2}$ for	GGTTTACGCCAAGTGGGCAGA
Rt-*SC*$_{1/2}$ rev	AGGGCAATACCGAAGCTGACC
Rt-*CA*$_{1/2}$ for	GCTGATTCGCCTTTGTCTAATA
Rt-*CA*$_{1/2}$ rev	CAGGATCCACACCAGCCAAAAC
Rt-*AB*$_{1/2}$ for	GTAACGATGGACGTACAAAATC
Rt-*AB*$_{1/2}$ rev	TTGTTAAAGGTACGCAGCAGCG
Rt-*BU*$_{1/2}$ for	ATGAGTAATGTAGTGACAGACC
Rt-*BU*$_{1/2}$ rev	TCGTTTTGATAATCGCCATCAC
Rt-*U3543*$_{1/2}$ for	TAATCTGAATTTAAACAAGCAC
Rt-*U3543*$_{1/2}$ rev	TATTCAGCGCTGGAAACCCCA

IV.2.5 Electrophoretic mobility shift assay

The full length PhoB protein was mixed with purified 5' 6-FAM labeled promoter region of *evpA* labelled with in a buffer containing 50 mM Tris-HCl, pH 7.5, 200 mM KCl, 0.1 mM EDTA and 5% v/v glycerol. The mixture was incubated at 25^{o}C for 2h before loading onto a non-denaturant 12% native polyacrylamide gel.

IV.2.6 Western blot analysis

Proteins were separated on 12 % SDS-polyacrylamide gels. For western analyses, proteins were transferred to a PVDF membrane as described in Chapter III.

IV.2.7 Preparation of TCPs and ECPs

Overnight cultures of *E. tarda* grown at different temperatures in DMEM (OD_{550}=0.8) were diluted 1:200 into fresh DMEM and incubated for 24 h at the indicated conditions. For the isolation of TCPs, the whole broth containing the cells were kept on ice for 5 mins to cool the contents and sonicated on ice for 5 min stopping every 1 min to agitate and reposition the sonicator probe. The above culture was then subjected to 10% TCA precipitation for 1 hour at 4°C , and the protein pellet was washed thrice with -20^{o}C acetone and then air-dried. ECP protein pellets were solubilized in Ready Prep reagent 3 (5 M urea, 2 M thiourea, 2 % (w/v) CHAPS, 2% (w/v) SB 3-10, 40 mM Tris, and 0.2% (w/v) Bio-Lyte 3/10 ampholyte (Bio-Rad, Hercules, CA)) and stored at -80^{o}C until analysis. The extraction of ECP was performed in the same way as described in Chapter III. The protein concentration was determined with a Bio-Rad protein assay kit using bovine serum albumin as the standard.

IV.2.8 Isolation of RNA and RT-PCR experiments

RNA was islaoted as described in Chapetr II. Six pairs of primers were designed based on the genomic DNA sequence. Pair A (Rt-$3537S_{1/2}$) was designed to amplify region from amino acid residue 200 of ETAE 3537 to residue 50 of PstS; pair B (Rt-$SC_{1/2}$) from residue 39 of PstS to residue 96 of PstC; pair C (Rt-$CA_{1/2}$) from residue 2 of PstC to residue 42 of PstA; pair D (Rt-$AB_{1/2}$) from residue 2 of PstA to residue 53 of PstB; pair E (Rt-$U3543_{1/2}$) from residue 2 of PstB to residue 53 of PhoU; pair F from residue 88 of PhoU to residue 137 of ETAE3543.

Table IV.2. Strains and plasmids for this study

Strain/plasmid	Description	Reference/Source
Bacteria		
E. tarda PPD130/91	Wild type, Km^s, Col^r, Amp^s, $LD_{50}=10^{5.0}$	(S. H. M. Ling, 2000)
ΔphoB	PPD130/91, in-frame deletion of *phoB* (missing amino acids 15 to 230)	This study
Δfur	PPD130/91, in-frame deletion of *fur* (missing amino acids 15 to 100)	This study
$phoU_i$	*phoU*::pRE112-phoU Cm^r	This study
$phoU_i$ + *phoU*	$phoU_i$ with pACYC + *phoU*	This study
$pRWphoB_{130/91}$	pRW50 containing -900 to +42 of *phoB* relative to putative translational start site in *E.tarda* PPD130/91	This study
$pRWfur_{130/91}$	pRW50 containing -801 to + 39 of *fur* relative to putative translational start in *E.tarda* PPD130/91	This study
$pRWesrB_{130/91}$	pRW50 containing -900 to +150 of *esrB* relative to putative translational start site in *E.tarda* PPD130/91	Chapter III
$pRWesrC_{130/91}$	pRW50 containing -852 to +51 of *esrC* relative to putative translational start site in *E.tarda* PPD130/91	This study
$PVIKphoU_{130/91}$	pVIK112, *phoU* internal fragment; in-frame transcriptional fusion *E.tarda* PPD130/91	This study
$pRWphoB_{\Delta phoB}$	pRW50 containing -900 to + 42 of *phoB* relative to putative translational start site in *E.tarda ΔphoB*	This study
$pRWphoB_{\Delta fur}$	pRW50 containing -801 to + 39 of phoB relative to putative translational start site in *E.tarda Δfur*	This study
$pRWphoB_{phoUi}$	pRW50 containing -900 to + 42 of phoB relative to putative translational start site in *E.tarda phoUi*	This study
$pRWfur_{\Delta phoB}$	pRW50 containing -801 to + 39 of fur relative to putative translational start in *E.tarda ΔphoB*	This study
$pRWfur_{\Delta fur}$	pRW50 containing -801 to + 39 of fur relative to putative translational start in *E.tarda Δfur*	This study
$pRWfur_{phoUi}$	pRW50 containing -801 to + 39 of fur relative to putative translational start in *E.tarda phoUi*	This study

pRWesrB$_{\Delta phoB}$	pRW50 containing -900 to +150 of esrB relative to putative translational start site in *E.tarda ΔphoB*	This study
pRWesrB$_{\Delta fur}$	pRW50 containing -900 to +150 of esrB relative to putative translational start site in *E.tarda Δfur*	This study
pRWesrB$_{phoUi}$	pRW50 containing -900 to +150 of esrB relative to putative translational start site in *E.tarda phoUi*	This study
pRWesrC$_{\Delta phoB}$	pRW50 containing -852 to +51 of esrC relative to putative translational start site in *E.tarda ΔphoB*	This study
pRWesrC$_{\Delta fur}$	pRW50 containing -852 to +51 of esrC relative to putative translational start site in *E.tarda Δfur*	This study
pRWesrC$_{phoUi}$	pRW50 containing-852 to +51of esrC relative to putative translational start site in *E.tarda phoUi*	This study
PVIKphoU$_{\Delta phoB}$	pVIK112, phoU internal fragment; in-frame transcriptional fusion in *E.tarda ΔphoB*	This study
PVIKphoU$_{\Delta fur}$	pVIK112, phoU internal fragment; in-frame transcriptional fusion in *E.tarda Δfur*	This study
PVIKphoU$_{phoUi}$	pVIK112, phoU internal fragment; in-frame transcriptional fusion in *E.tarda phoUi*	This study
E. coli		
JM109	Km^{s}, Col^{s}, Cm^{s}	Promega
MC1061 (λpir)	thi thr1 leu6 proA2 his4 argE2 lacY1 galK2 ara14 xyl5 supE44 pir	(Rubirés, 1997)
SM10 (λpir)	thi thr leu tonA lacY supE recA-RP4-2-Tc-Mu Km^{r} pir	(Rubirés, 1997)
BL21 (DE-3)	genotype: F^{-} *omp*T *hsd*S$_B$(r$_B^-$m$_B^-$) *gal dcm*	Stratagene
S17-1 (λpir)	Tp^{r} Sm^{r} recA, thi, pro, hsdR-M^{+} RP4: 2-Tc:Mu: Km Tn7 λpir	(Simon, 1983)
pETM-phoB$_s$	Full length PhoB Bl-21 cells cloned in pETM	This study
Plasmid		
pGEM-T Easy	Amp^{r}	Promega

pRE112	pGP704 suicide plasmid, pir dependent, Cm^r, oriT, oriV, sacB	(Edwards, 1998)
pACYC184	Tet^r, Cm^r	Amersham
pRW50	lacZ reporter vector, Tet^r	(Lodge, 1992)
pETM	modified pET-32a plasmid	Novagen

IV.3 Results

IV.3.1 Identification of two-component regulatory system PhoB-PhoR

Using the genome sequence of *Edwardsiella tarda EIB202* (Wang et al, 2009a) and related bacteria as the template, degenerate primers were designed based on the conserved nucleotide sequences to identify the *phoB* and *phoR* genes of *E. tarda* PPD130/91. Sequence alignment of PhoR and PhoB of *E. tarda* showed a 70% and 84% identity to *Yersinia pestis KIM* (Deng et al, 2002b), followed by a 68% and 83% identity to both *Salmonella typhimurium LT2* (McClelland et al, 2001b) and *Escherichia coli CFT073* (Welch et al, 2002b) respectively. In addition, the sequence identities of both PhoR and PhoB between *E. tarda* EIB20*2* and *E. tarda* PPD130/91 are 100 % and 99.0%, respectively (Fig. IV.1 and Fig. IV.3) The high sequence identity of PhoR and PhoB between the two different species of *E. tarda* suggests similar and conserved functions of the proteins among different strains of *E. tarda*. In contrast, particularly for PhoR, the relatively low sequence identity was observed between *E. tarda* 130/91 and those of *E.coli* and *S. typhimurium*, suggesting diverse functions of the sensor protein PhoR. The predicted secondary structure of PhoR and PhoB is shown in Fig. IV.2 and Fig. IV.4) respectively.

```
PhoR_ST           ---VLERLSWKRLVLELVLCCIPALILSAFFGYLPWFLLAAVTGLLVWHFWNLLRLSWWL
PhoR_EC           ---VLERLSWKRLVLELLLCCLPAFILGAFFGYLPWFLLASVTGLLIWHFWNLLRLSWWL
PhoR_YP           ---VLERLSWKTLALELALFCLPALLLGAFIGYLPWLLLVSVVAALTWNFYNQLKLSHWL
PhoR_EIB202       ---VLDKLSWKTLLSELLLCCVPALLLGALFGYLPWFLLSALCLLLLWHGWNQLRLSHWL
PhoR_ET130/91     VLTVLDKLSWKTLLSELLLCCVPALLLGALFGYLPWFLLSALCLLLLWHGWNQLRLSHWL
                     **::**** *  ** * *:**::*.*::*****:** ::   * *: :* *:** **

PhoR_ST           WVDRSMTPPPGRGSWEPLLYGLHQMQLRNKKRRRELGNLIKRFRSGAESLPDAVVLTTEE
PhoR_EC           WVDRSMTPPPGRGSWEPLLYGLHQMQLRNKKRRRELGNLIKRFRSGAESLPDAVVLTTEE
PhoR_YP           WLDRSMTPPSGRWSWEPLFYGLYQMQLRNRRRRRELALLIKRFRSGAESLPDAVVITTVD
PhoR_EIB202       WVDRSMTPPSGRGSWEPLFYGLYQMQQRNRRRRRELALLIKRFRSGAESLPDAIVMLTDE
PhoR_ET130/91     WVDRSMTPPSGRGSWEPLFYGLYQMQQRNRRRRRELALLIKRFRSGAESLPDAIVMLTDE
                  *:*******.** *****:***:*** **::*****. ***************:*: * :

PhoR_ST           GGIFWCNGLAQQILGLRWPDDNGQNILNLLRYPEFTQYLKTRDFTRPLHLVLNTGRHLEI
PhoR_EC           GGIFWCNGLAQQILGLRWPEDNGQNILNLLRYPEFTQYLKTRDFSRPLNLVLNTGRHLEI
PhoR_YP           GNIFWCNGLAQQLLGFRWPEDNGQHILNLLRYPEFSQYLQQQTFSRPLTLQLNNGYYVEF
PhoR_EIB202       GNIFWCNHLAQHLLGFRWPEDNGQNIRNLLRYPEFSRYLGDADYARPLTLHLNSGRYMEF
PhoR_ET130/91     GNIFWCNHLAQHLLGFRWPEDNGQNIRNLLRYPEFSRYLGDADYARPLTLHLNSGRYMEF
                  *.***** ***::**:***:****:* ********::**    ::*** * **.* ::*:

PhoR_ST           RVMPYTDKQLLMVARDVTQMHQLEGARRNFFANVSHELRTPLTVLQGYLEMMQEQALEGA
PhoR_EC           RVMPYTHKQLLMVARDVTQMHQLEGARRNFFANVSHELRTPLTVLQGYLEMMDEQPLEGA
PhoR_YP           RVMPYSEGQLLMVARDVTQMRQLEGARRNFFANVSHELRTPLTVLQGYLEMMHDQELAGP
PhoR_EIB202       RLMPYSEGQMLMIARDVTQKRQLEGARRNFFANVSHELRTPLTVLQGYLEMFGDDAMPAP
PhoR_ET130/91     RLMPYSEGQMLMIARDVTQKRQLEGARRNFFANVSHELRTPLTVLQGYLEMFGDDAMPAP
                  *:***:. *:**:****** :*****************************: :: : ..

PhoR_ST           TREKALHTMREQTYRMEGLVKQLLTLSKIEAAPALLLNEKVDVPMMLRVVEREAQTLSQQ
PhoR_EC           VREKALHTMREQTQRMEGLVKQLLTLSKIEAAPTHLLNEKVDVPMMLRVVEREAQTLSQK
PhoR_YP           LRDKALGTMQEQTKRMDGLVKQLLTLSRIEAAPNVDLNERVDIPLMLKILQHEVQALSNG
PhoR_EIB202       QREKALLTMQAQAQRMDGLVRQLLVLSRIEAAPDIDLSAVVDVPAMLHMLEREANALSDG
PhoR_ET130/91     QREKALLTMQAQAQRMDGLVRQLLVLSRIEAAPDIDLSAVVDVPAMLHMLEREANALSDG
                   *:*** **: *: **:***:***.**:*****   *.  **:* **:::::*.::**:

PhoR_ST           KHTFTFEVDDSLSVLGNEEQLRSAISNLVYNAVNHTPAGTHITVSWRRVAHGAEFCIQDN
PhoR_EC           KQTFTFEIDNGLKVSGNEDQLRSAISNLVYNAVNHTPEGTHITVRWQRVPHGAEFSVEDN
PhoR_YP           RHEITFRVNDQLKVFGNEDQLRSAVSNLVYNAVNHTPEGTKIEVCWQQTPQGAQFQVSDN
PhoR_EIB202       QHTLRFSIDDGLRVRGNEEQLRSAVSNLVYNAINHTPAGTQIRVCWQRTAQGAYFSVQDN
PhoR_ET130/91     QHTLRFSIDDGLRVRGNEEQLRSAVSNLVYNAINHTPAGTQIRVCWQRTAQGAYFSVQDN
                  :: : * ::: * * ***:*****:*******:**** **:* * *::..:** * :.**

PhoR_ST           GPGIAAEHIPRLTERFYRVDKARSRQTGGSGLGLAIVKHALNHHESRLEIDSSPGKGTRF
PhoR_EC           GPGIAPEHIPRLTERFYRVDKARSRQTGGSGLGLAIVKHAVNHHESRLNIESTVGKGTRF
PhoR_YP           GPGIPPEHVPRLTERFYRVDKARSRQTGGSGLGLAIVKHALSHHDARLEVMSEIGLGTRF
PhoR_EIB202       GPGIAQEHLSHLTERFYRVDRSRSSRTGGSGLGLAIVKHALQHHEAQLAIDSALGQGTRF
PhoR_ET130/91     GPGIAQEHLSHLTERFYRVDRSRSSRTGGSGLGLAIVKHALQHHEAQLAIDSALGQGTRF
                  ****. **:.:*********::** :**************:.**:::* : *  * ****

PhoR_ST           SFVLPERLIAKNSD-------
PhoR_EC           SFVIPERLIAKNSD-------
PhoR_YP           IFTLPNRLIVPTVLVENAVKS
PhoR_EIB202       AFTLPSHLVFNGAPSA-----
PhoR_ET130/91     AFTLPSHLVFNGAPSA-----
                   *.:*.:*:
```

Fig. IV.1 Amino acid sequence alignment of PhoR *E. tarda* 130/91 with related bacterial species. The (⋆) indicates identical residues and (:) denotes similar residues.

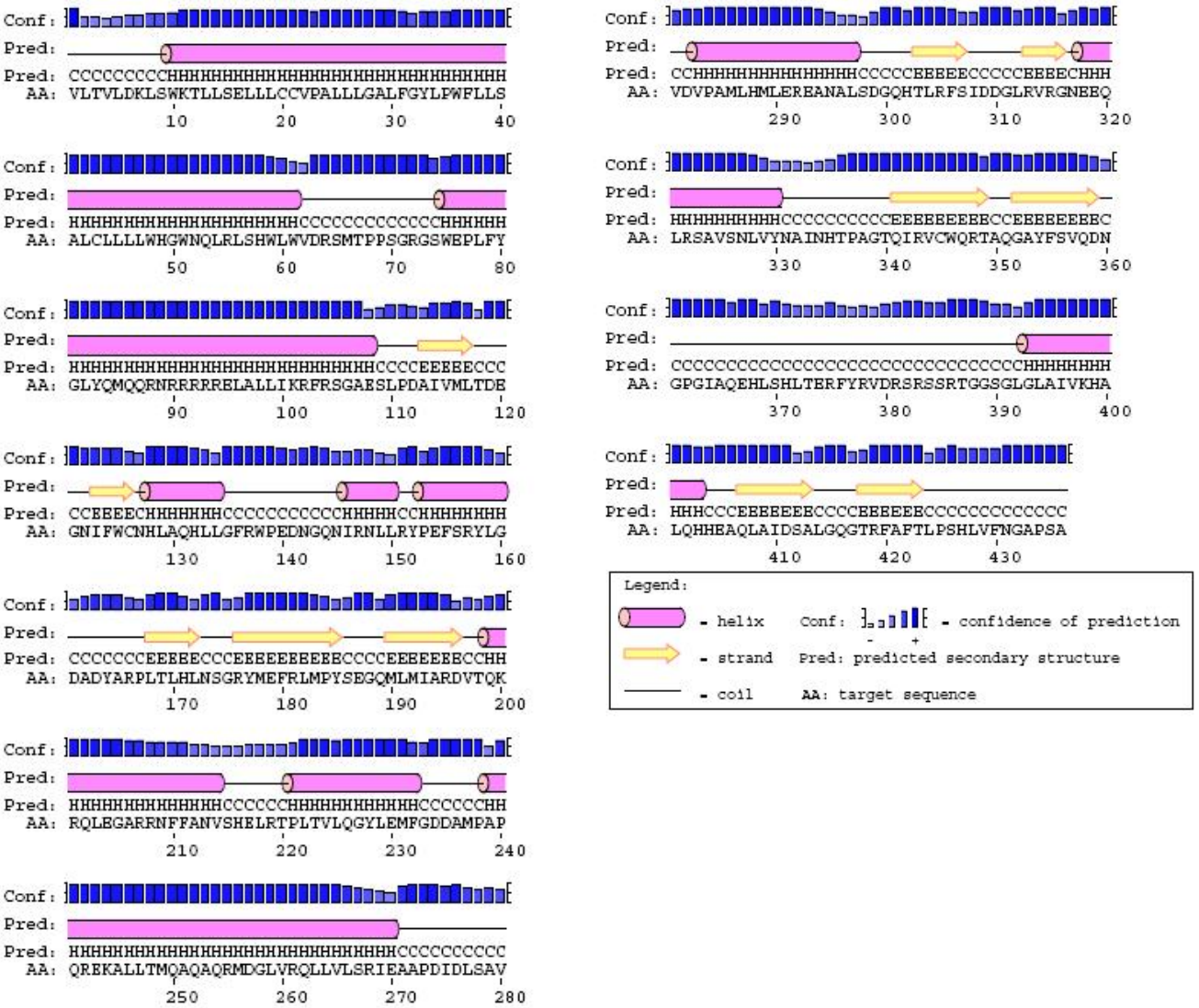


Fig. IV.2 Predicted secondary structure of PhoR *E. tarda* 130/91

```
PhoB_ST           -----------ARRILVVEDEAPIREMVCFVLEQNGFQPVEAEDYDSAVNKLNEPWPDLI
PhoB_EC           ----------MARRILVVEDEVPIREMVCFVLEQNGFQPVEAEDYDSAVNQLNEPWPDLI
PhoB_YP           VTANILAGLMMARRILVVEDEAPIREMVCFVLEQNGYQPLEAEDYDSAVARLSEPFPDLV
PhoB_EIB_202      ----------MSIRILVVEDETPIRDMVSFVLEQNGYQPLEAESYDGALSQLCEPYPDLI
PhoB_ET130/91     ----------MTIRILVVEDETPIRDMVSFVLEQNGYQPLEAESYDGALSQLCEPYPDLI
                            : ********.***:**.*******:**:***.**.*: :* **:***:

PhoB_ST           LLDWMLPGGSGLQFIKHLKREAMTRDIPVVMLTARGEEEDRVRGLETGADDYITKPFSPK
PhoB_EC           LLDWMLPGGSGIQFIKHLKRESMTRDIPVVMLTARGEEEDRVRGLETGADDYITKPFSPK
PhoB_YP           LLDWMLPGGSGIQFIKHMKREALTRDIPVMMLTARGEEEDRVRGLEVGADDYITKPFSPK
PhoB_EIB_202      LLDWMLPGGSGIQLIKQLKREPATREIPVMMLTARGEEEDRVRGLEVGADDYITKPFSPK
PhoB_ET130/91     LLDWMLPGGSGIQLIKQLKREPATREIPVMMLTARGEEEDRVRGLEVGADDYITKPFSPK
                  ***********:*:**::***. **:***:****************.*************

PhoB_ST           ELVARIKAVMRRISPMAVEEVIEMQGLSLDPGSHRVMTGDSPLDMGPTEFKLLHFFMTHP
PhoB_EC           ELVARIKAVMRRISPMAVEEVIEMQGLSLDPTSHRVMAGEEPLEMGPTEFKLLHFFMTHP
PhoB_YP           ELVARIKAVMRRISPMAVEEVIEMQGLSLDPSSHRVMANDQALDMGPTEFKLLHFFMTHP
PhoB_EIB_202      ELVARIKAVMRRISPMALEETINLQGLSLDPVSHRVTAQDDAVEMGPTEFKLLHFFMTHP
PhoB_ET130/91     ELVARIKAVMRRISPMALEETINLQGLSLDPVSHRVTAQDDAVEMGPTEFKLLHFFMTHP
                  *****************:**.*::******* **** : :..::***************

PhoB_ST           ERVYSREQLLNHVWGTNVYVEDRTVDVHIRRLRKALEHSGHDRMVQTVRGTGYRFSTRF
PhoB_EC           ERVYSREQLLNHVWGTNVYVEDRTVDVHIRRLRKALEPGGHDRMVQTVRGTGYRFSTRF
PhoB_YP           ERVYSREQLLNYVWGTNVYVEDRTVDVHIRRLRKALETDGHDKMVQTVRGTGYRFSTRY
PhoB_EIB_202      ERVYSREQLLNHVWGTNVYVEDRTVDVHIRRLRKALEGSGHDRMVQTVRGTGYRFSARF
PhoB_ET130/91     ERVYSREQLLNHVWGTNVYVEDRTVDVHICRLRKALEGSGHDRMVQTVRGTGYRFSARF
                  ***********:**************** ******* .***:*************:*:
```

Fig. IV.3 Amino acid sequence alignment of PhoB *E. tarda* 130/91 with related bacterial species. The (⋆) indicates identical residues and (:) denotes similar residues.

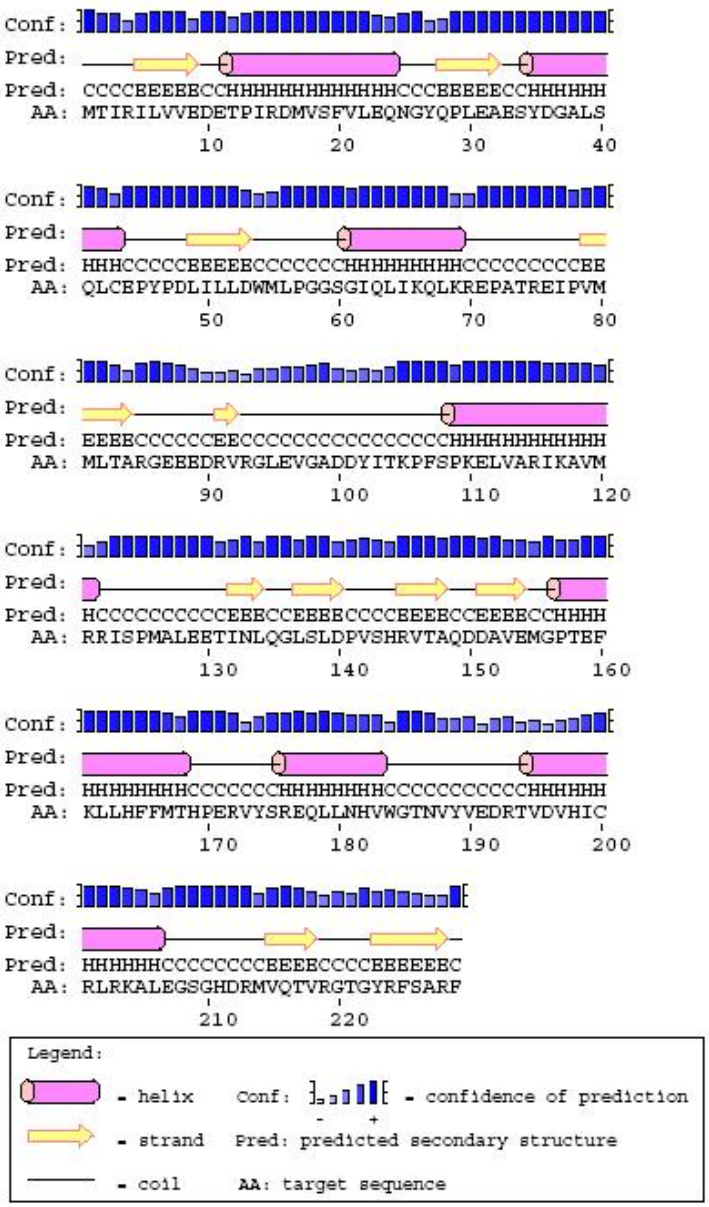
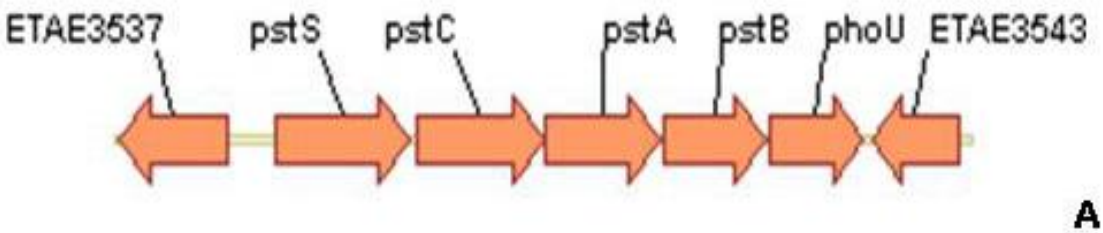


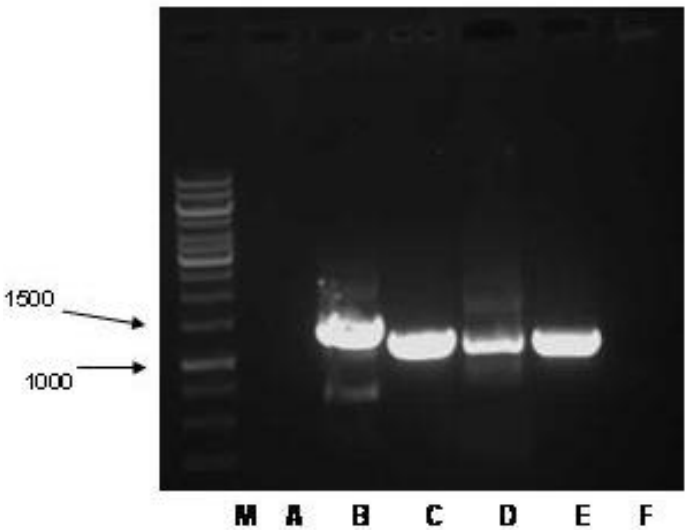
Fig. IV.4 Predicted secondary structure of PhoB *E. tarda* PPD 130/91

IV.3.2 *pstSCAB-phoU* operon is polycistronic and induced under low phosphate and low iron conditions.

Using RT-PCR experiments on RNA isolated from *E. tarda* PPD130/91, all the five genes belonging to the *pstSCAB-phoU* operon were found to be co-transcribed as a single operon. Six pairs of primers were designed based on the genomic DNA sequence. Pair A was designed to amplify region from amino acid residue 200 of ETAE 3537 to residue 50 of PstS; pair B from residue 39 of PstS to residue 96 of PstC; pair C from residue 2 of PstC to residue 42 of PstA; pair D from residue 2 of PstA to residue 53 of PstB; pair E from residue 2 of PstB to residue 53 of PhoU; pair F from residue 88 of PhoU to residue 137 of ETAE3543. Except primers A and E, the rest produced bands of correct size indicating co-transcription of *pstS*, *pstC*, *pstA*, *pstB* and *phoU* (Fig. IV.5). To study the effects of phosphate and Fe^{2+} concentration on the expression levels of the polycistronic *pstS* promoter, strain harboring lacZ fusion genes under the control of the putative promoter for *pstS* was created and the ß-galactosidase assay was conducted at the indicated conditions. The expression of *pstS* was reduced to 41.6% and 33.3% in the presence of phosphate and iron, respectively. Maximum suppression of *pstS* expression by 83.3% was observed in the presence of both phosphate and iron together (Fig. IV.7). Since environmental factors such as high iron and high phosphate reduced transcription of *pstSCAB-phoU* operon, secretion proteins from the T3SS and T6SS of wild type bacteria was studied in the presence of iron and phosphate.



A



Primers	Length of DNA fragment to be amplified (bp)
Pair A	1425
Pair B	1295
Pair C	1117
Pair D	1109
Pair E	1085
Pair F	1046

B

Fig. IV.5 Genomic organization and co-transciption of *pstSCAB-phoU* as a single operon. (A)Genetic organization of *pstSCAB-phoU* operon. (B) RT-PCR experiments were performed using RNA isolated from *E. tarda* PPD130/91 and primers are listed in (Table IV.1). Pair A was designed to amplify region from amino acid residue 276 of ETAE3537 to residue 75 of PstS; pair B from residue 37 of PstS to residue 103 of PstC; pair C from residue 2 of PstC to residue 42 of PstA; pair D from residue 2 of PstA to residue 52 of PstB; pair E from residue 1 of PstB to residue 88 of PhoU; pair F from residue 2 of PhoU to residue 170 of ETAE3543.

IV.3.3 Environmental factors such as phosphate and Fe^{2+} concentrations affect the secretion and expression of T3SS and T6SS proteins in wild type *E. tarda* PPD130/91

To investigate the effects of phosphate and Fe^{2+} concentration on the secretion of T3SS and T6SS proteins, *E. tarda* wild type bacterial cultures were grown in DMEM at an ambient temperature of 30 ^{o}C supplemented with 20mM KH_2PO_4 and /or 20μM $FeSO_4$ as indicated and the amount of secreted ECPs were quantified. SDS-PAGE analysis showed that secretion of both T3SS (EseB, EseC and EseD) and T6SS (EvpC and EvpP) proteins were highly suppressed in the presence of both phosphate and Fe^{2+} (ECP= 1.0 ± 0.04 μg/ml) (Fig. IV.6). Maximum secretion of ECP was observed in absence of both phosphate and Fe^{2+} (ECP=3.00 ± 0.12 μg/ml). Presence of either phosphate or Fe^{2+} also reduced the secretion of proteins where greater suppressing effect was observed in the presence of Fe^{2+} in comparison to that of phosphate (ECP in 20mM KH_2PO_4=2.25 ± 0.07 μg/ml; ECP in 20μM $FeSO_4$ =1.5 ± 0.03 μg/ml). In wild type *E. tarda*, the transcription of *esrC* was suppressed by 91% in presence of both phosphate and iron but not *esrB* based on the LacZ assay. (Fig IV.7). In presence of only phosphate, at least 50% reduction was observed in the expression of *phoB*, *pstS* and *phoU*, suggesting that *pstSCAB-phoU* and *phoBR* operon are only triggered under phosphate starvation condition. Almost 40% reduction in expressing of *phoB* and *phoU* was also observed in the presence of iron (Fig. IV.7). This indicates that iron by itself negatively affects *phoB* as well as *pstSCAB-phoU* operon. Both iron and phosphate exert an additive effect on the secretion of T3SS and T6SS proteins in *E. tarda* PPD130/91.

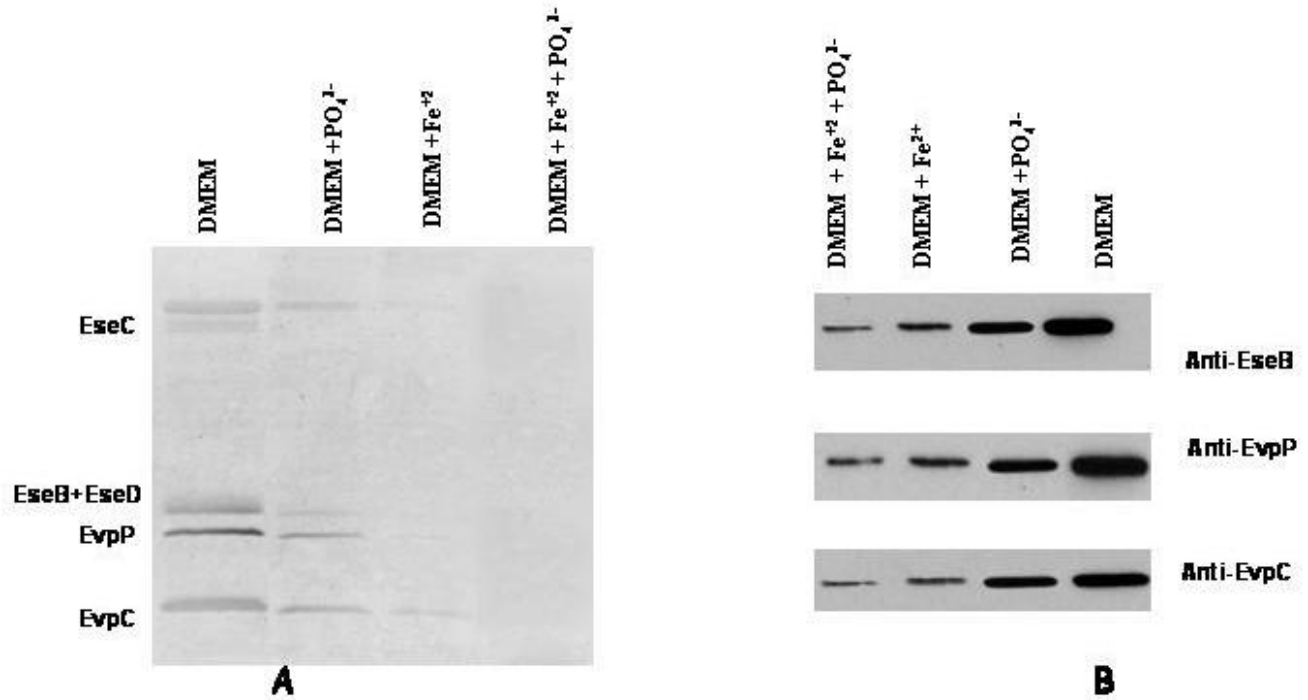


Fig. IV.6. **Additive effect of phosphate and iron on the expression and secretion of T3SS and T6SS proteins in *E. tarda* PPD 130/91**. Silver stained SDS PAGE showing T3SS and T6SS ECPs secretion (A) and (B) Western blot analysis of TCP showing the expression of EseB from T3SS; EvpP and EvpC from T6SS by *E. tarda* PPD130/91 cultured in DMEM in presence or absence of phosphate and/or iron as indicated. Both the ECP and TCP fractions were adjusted for equal number of bacterial cells as determined by standard plate count.

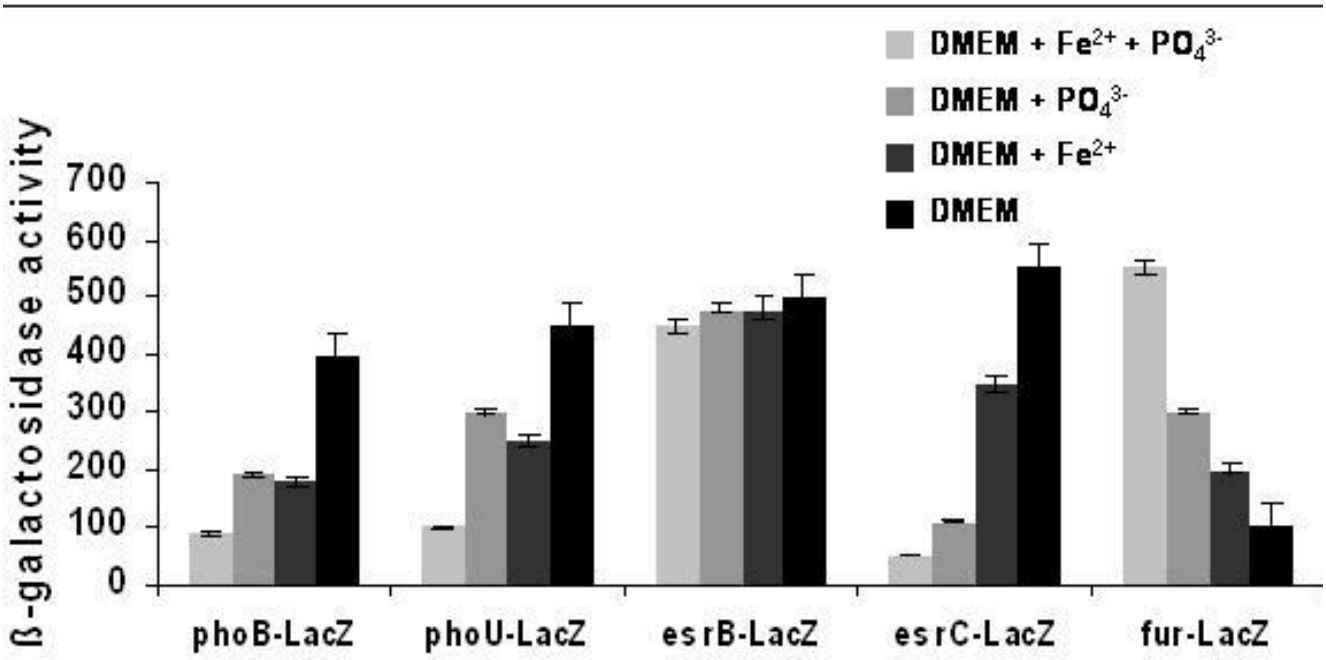
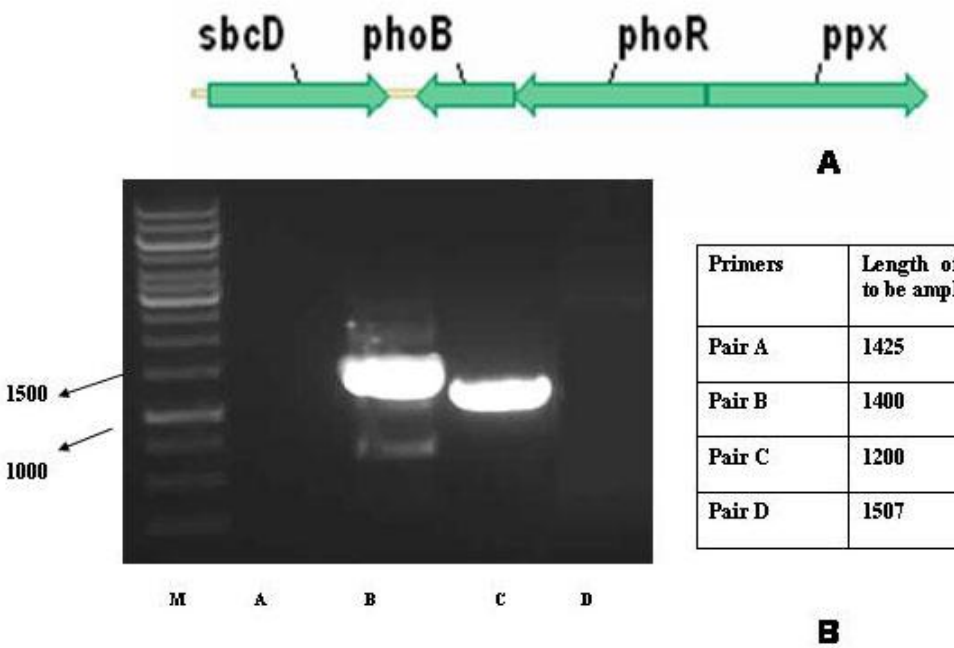


Fig. IV.7. **Effect of phosphate and iron on the expression of *phoB*, *phoU*, *esrB*, *esrC* and *fur* in *E. tarda* PPD 130/91**. The ß-galactosidase activities of the reporter genes *phoB*-LacZ, *phoU*-LacZ, *esrB*-LacZ and *esrC*-LacZ in *E. tarda* PPD30/91 were determined in DMEM in presence or absence of iron and/or phosphate as indicated. The values represent the mean ± SD from one representative experiment performed with triplicate samples. Equivalent results were obtained at least in triplicates.

IV.3.4 PhoB positively regulates the secretion of EvpC by binding to the promoter region of *evpA* and functions through EsrC.

A sequence motif [CTGTCATxxxxCTGTCAT] comprising a repeat of two hexanucleotide sequences separated by five bases has been defined as the high affinity PhoB binding region named as "Pho box" in which the second half of the box is highly degenerate. Inspection of the entire genome sequence of the closely related *E. tarda* EIB202 with the criteria of maximum two degeneracy in the second half of the pho box and a minimum promoter prediction score of 0.5, identified putative PhoB binding sites in the promoter regions of *phoB*, *pstS* and *evpA*. Electrophoretic mobility shift assay showed that the His-tagged DNA binding domain (DBD) of PhoB can bind to the 450-bp from the promoter region of *evpA*. To investigate a possible contribution of PhoB to the virulence of *E. tarda*, non-polar deletion mutant of *phoB* (Δ*phoB*) was constructed. The growth and protein secretion of the wild type *E. tarda* PPD130/91 and Δ*phoB E. tarda* were measured at 30^{o}C in presence or absence of phosphate and/or iron. SDS-PAGE analysis showed that the Δ*phoB* mutant was deficient in the secretion of EvpC in spite of having comparable cell density with that of wild type bacteria (Fig. IV.11). The *evpA* gene in the type VI cluster (Fig. IV.10) is present upstream of *evpC* which is homologous to hemolysin co-regulated protein (Hcp) and valine glycine repeat protein (VgrG) (Bingle et al, 2008;Filloux et al, 2008;Pukatzki et al, 2009)



Primers	Length of DNA fragment to be amplified (bp)
Pair A	1425
Pair B	1400
Pair C	1200
Pair D	1507

Fig. IV.8. Genetic organization and co-transcription of *phoB/phoR* as a single operon. (A)Genetic organization of *phoBR* operon. (B). RT-PCR experiments were performed using RNA isolated from *E. tarda* PPD130/91 and four pairs of primers were designed based on the genomic DNA sequence listed in (Table IV.1). Pair A was designed to amplify region from amino acid residue 270 of SbcD to residue 75 of PhoB; pair B from residue 36 of PhoB to residue 95 of PhoR; pair C from residue 66 of PhoB to residue 120 of PhoR; pair D from residue 10 of PhoR to residue 145 of Ppx.

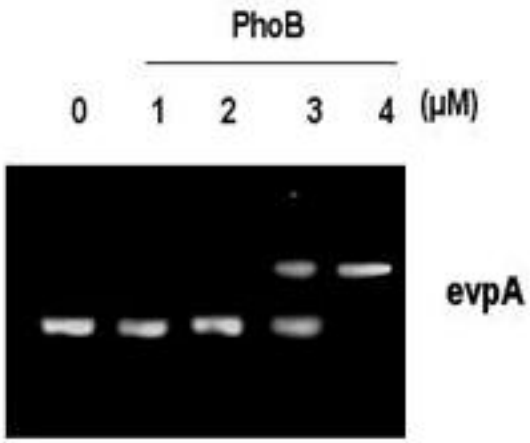


Fig. IV.9. EMSA of DBD of PhoB binding to the selected promoter regions. Electrophoretic mobility shift assay of PhoB DBD on a 5' 6-FAM labeled DNA fragment (-375 bp, from nt -360 to +15) from the promoter region of *evpA*. The DNA binding domain of PhoB directly bound to the promoter region of *evpA*.

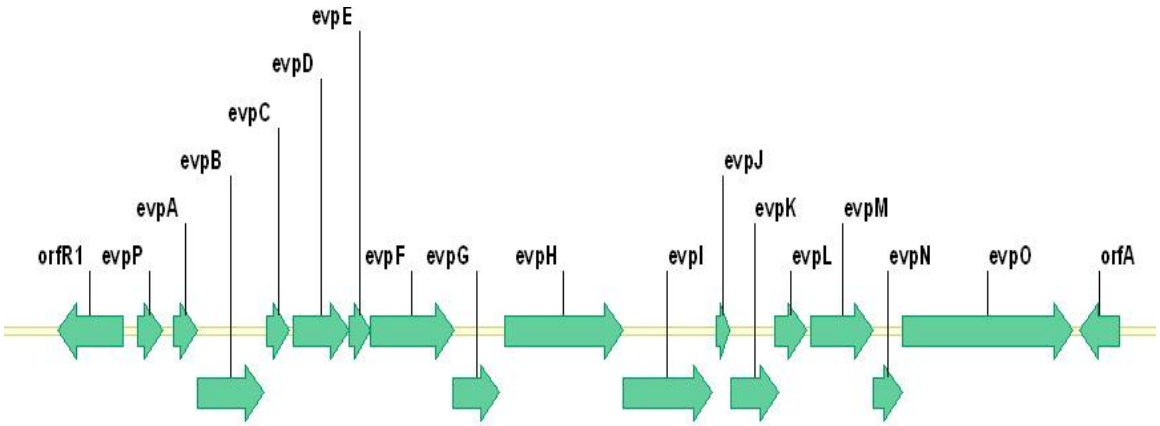


Fig. IV.10. Genetics organization of the T6SS gene cluster and its flanking ORFs.

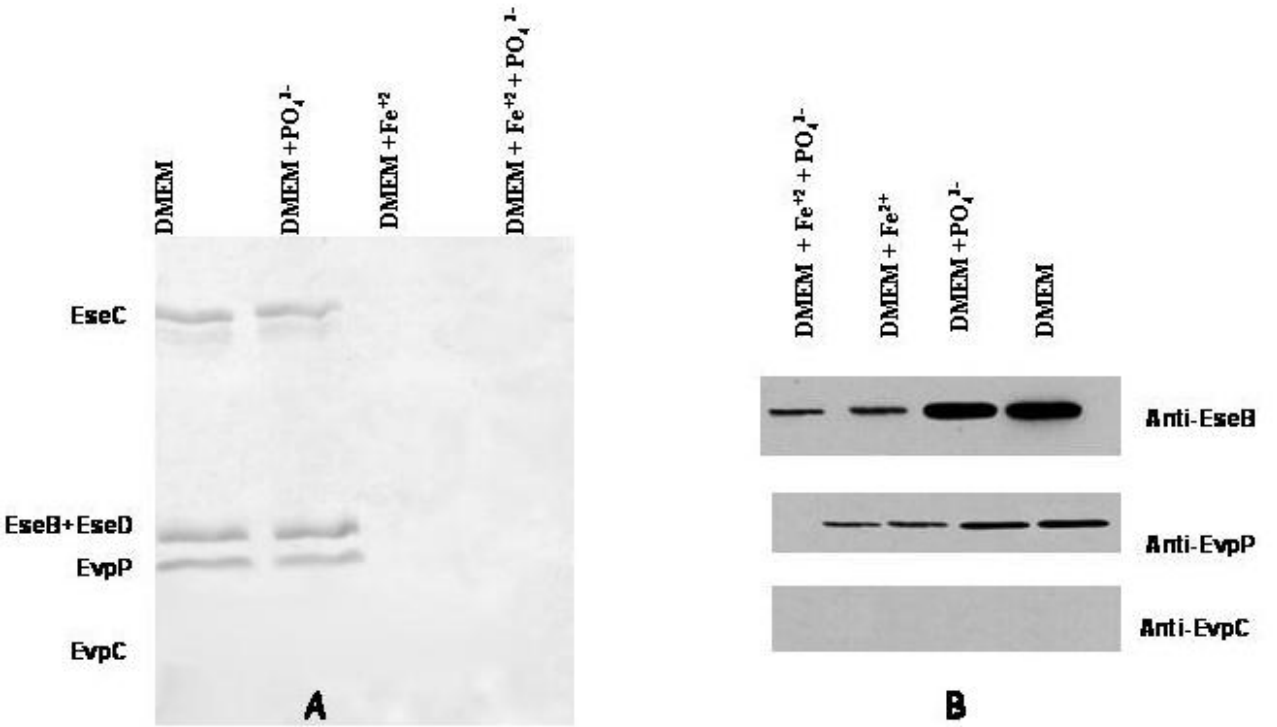


Fig. IV.11. **Additive effect of phosphate and iron on the expression and secretion of T3SS and T6SS proteins in *E. tarda ΔphoB***. Silver stained SDS PAGE showing T3SS and T6SS ECPs secretion (A) and (B) Western blot analysis showing the expression of EseB from T3SS; EvpP and EvpC from T6SS by *E. tarda ΔphoB* cultured in DMEM in presence or absence of phosphate and iron as indicated. Both the ECP and TCP fractions were adjusted for equal number of bacterial cells as determined by standard plate count.

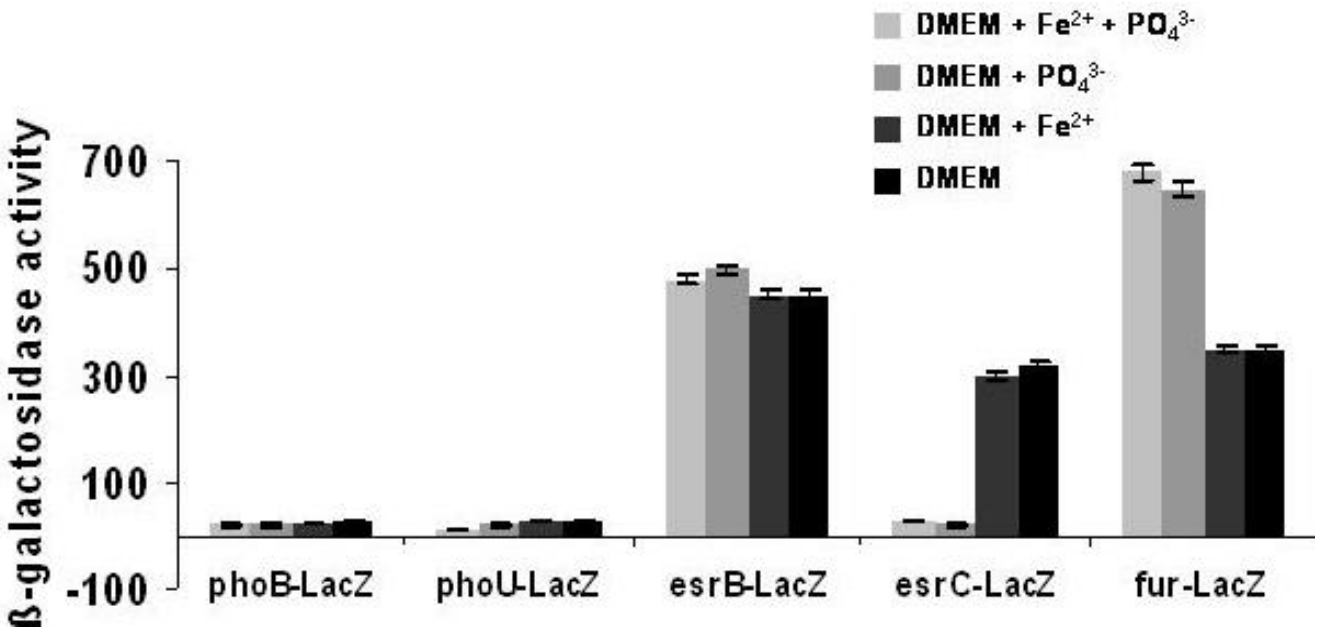
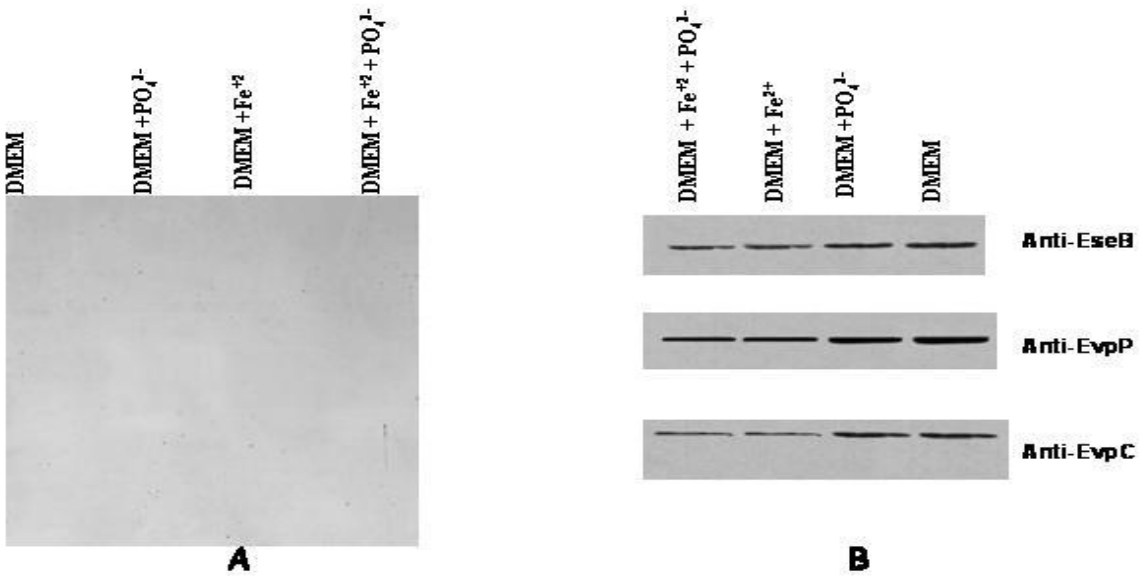


Fig. IV.12. **Effect of phosphate and iron on the expression of *phoB*, *phoU*, *esrB*, *esrC* and *fur* in *E. tarda ΔphoB*.** The ß-galactosidase activities of the reporter genes *phoB*-LacZ, *phoU*-LacZ, *esrB*-LacZ and *esrC*-LacZ were determined in *E. tarda ΔphoB* in DMEM in presence or absence of iron and/or phosphate as indicated. The values represent the mean ± SD from one representative experiment performed with triplicate samples. Equivalent results were obtained at least in triplicates.

The *ΔphoB* mutant of *E. tarda* showed no expression of EvpC. No changes in the secretion of ECP was observed in presence (ECP=2.25 ± 0.07 μg/ml) or absence of phosphate (ECP=2.3 ± 0.10 μg/ml). In contrast, Δ*phoB* still responds to changes in the iron concentration at levels comparable to the wild type (Fig. IV.11). In the TypeVI gene cluster, genes from *evpA* to *evpO* are regulated under the same promoter. PhoB binding to the promoter of *evpA* can only regulate the secretion of EvpC which is present downstream of *evpA* but not *EvpP* which is present upstream of *evpA* (Fig. IV.10). In Δ*phoB*, the expression of esrC was suppressed by 41.8% in comparison to wild type. In contrast, no reduction in the expression of *esrB* in the Δ*phoB* mutant bacteria was observed which suggests that PhoB can only work through *esrC* but not *esrB* (Fig. IV.12). In addition, high suppression of *phoU*-LacZ, *pstS*-LacZ and *phoB*-LacZ were observed confirming PhoB binding to the polycistronic promoter present upstream of *pstS* and regulating expression of *pstSCAB-phoU* operon. ECP profile of Δ*phoB* did not show any changes on addition of phosphate, indicating that PhoB/PhoR two component system is sensing phosphate for regulation of T3SS and T6SS secreted proteins. Transcriptional auto-regulation is possible in *phoBR* operon since PhoB binds to its own promoter and RT-PCR results also suggest that both *phoB* and *phoR* are co-transcribed (Fig. IV.8).

IV.3.5 PhoU positively controls T3SS and T6SS through EsrC

Insertional mutant of *phoU* (*phoUi*) was created to identify its possible function in the virulence of *E. tarda*. The growth and protein secretion profiles of the wild type *E. tarda* PPD130/91 and *phoUi* were measured at 30^{o}C in presence or absence of phosphate and/ or iron. SDS-PAGE analysis showed that *phoUi E. tarda* mutant was deficient in the secretion of



T6SS proteins in *E. tarda phoU$_i$*. Silver stained SDS PAGE showing T3SS and T6SS ECPs secretion (A) and (B) Western blot analysis showing the expression of EseB from T3SS; EvpP and EvpC from T6SS by *E. tarda phoU$_i$* cultured in DMEM in presence or absence of phosphate and iron as indicated. Both the ECP and TCP fractions were adjusted for equal number of bacterial cells as determined by standard plate count.

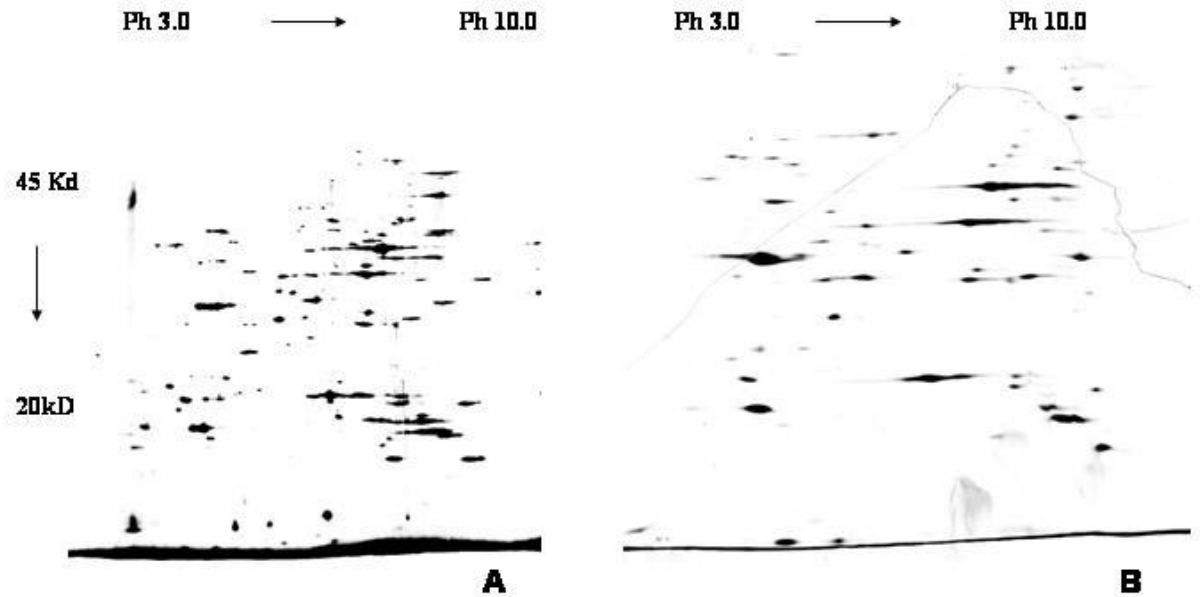


Fig. IV.14. Proteome analysis of *E. tarda* PPD 130/91 and the *E. tarda phoU$_i$* mutant. Total cellular protein (A) wild type; and (B) *phoU$_i$* mutant fractions in DMEM were separated on Immobiline DryStrips (pH 3-10) combined with 2D-PAGE analysis (12.5% polyacrylamide). Gels were silver stained.

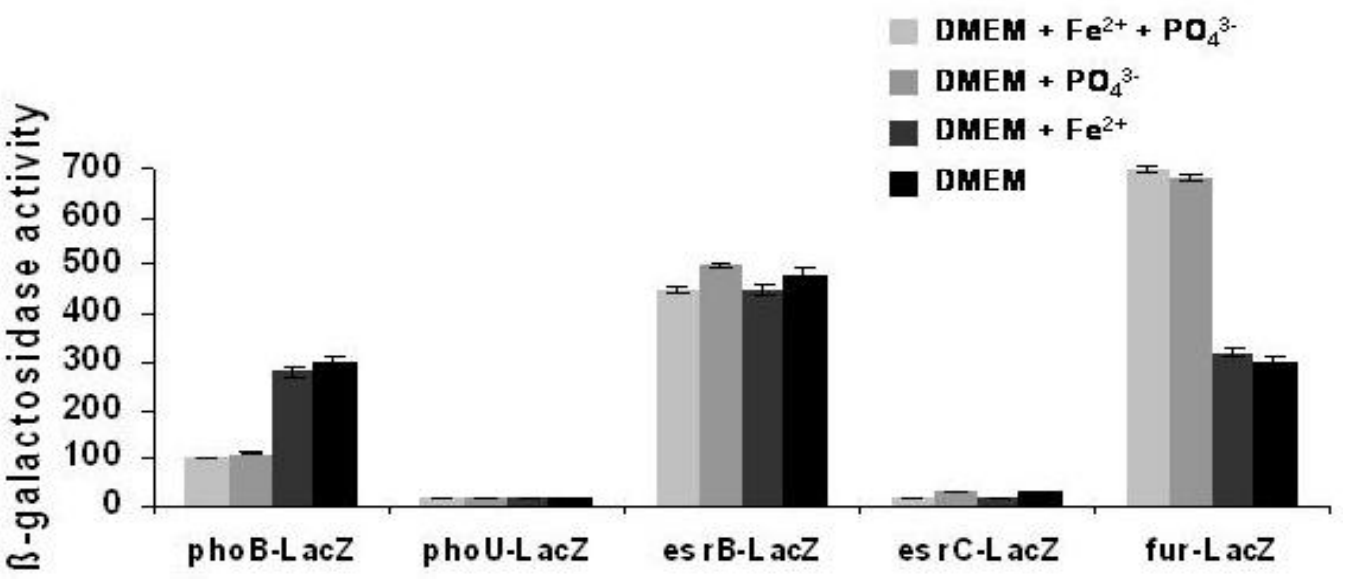


Fig. IV.15. **Effect of phosphate and iron on the expression of *phoB*, *phoU*, *esrB*, *esrC* and *fur* in *E. tarda phoU*$_i$.** The ß-galactosidase activities of the reporter genes *phoB*-LacZ, *phoU*-LacZ, *esrB*-LacZ and *esrC*-LacZ were determined in *E. tarda phoU*$_i$ in DMEM in presence or absence of iron and/or phosphate as indicated. The values represent the mean ± SD from one representative experiment performed with triplicate samples. Equivalent results were obtained at least in triplicates.

of T3SS as well as T6SS, proteins, even in the absence of both iron and phosphate (ECP=0.75 ± 0.10 μg/ml, *n*=3). Complete absence of both T3SS and T6SS secreted proteins was found in the supernatant of mutant bacteria *phoUi* suggesting a regulatory function of PhoU (Fig. IV.13). Total cell protein analysis of *E. tarda* PPD130/91 and *phoUi* mutant separated on Immobiline DryStrips (pH 3 to 10) combined with 2-DE analysis (12.5% polyacrilamide) identified suppression of several protein spots in the *phoUi* mutant bacteria also suggest additional regulatory role of PhoU besides phosphate uptake (Fig. IV.14). To identify the mode of function of PhoU, β-galactosidase activities of the reporter genes *phoB*-LacZ, *pstS*-LacZ, *phoU*-LacZ, *esrB*-LacZ and *esrC*-LacZ were determined in DMEM in presence or absence of iron and phosphate in *phoUi* mutant background. Mutant *phoUi* shows 94.5% reduction in the expression of *esrC*-LacZ in comparison to the wild type. In contrast, expression of *esrB*-LacZ in *phoUi* remains at similar level as wild type bacteria (Fig. IV.15). Our results suggest that PhoU exert its function through modulation of *esrC* but not *esrB*. In addition, PhoU may not act as a negative repressor of pho regulon in *E. tarda* since *phoUi* shows 41% reduction in the expression of *phoB*-LacZ in comparison with the wild type.

IV.3.6 Fur acts as a negative regulator of T3SS and T6SS; binds to *evpP* promoter and functions through EsrC.

Recently, ferric uptake regulator (Fur) has been identified as a link between environmental iron concentration and virulence in *E. tarda*. Generally, Fur protein binds to a consensus

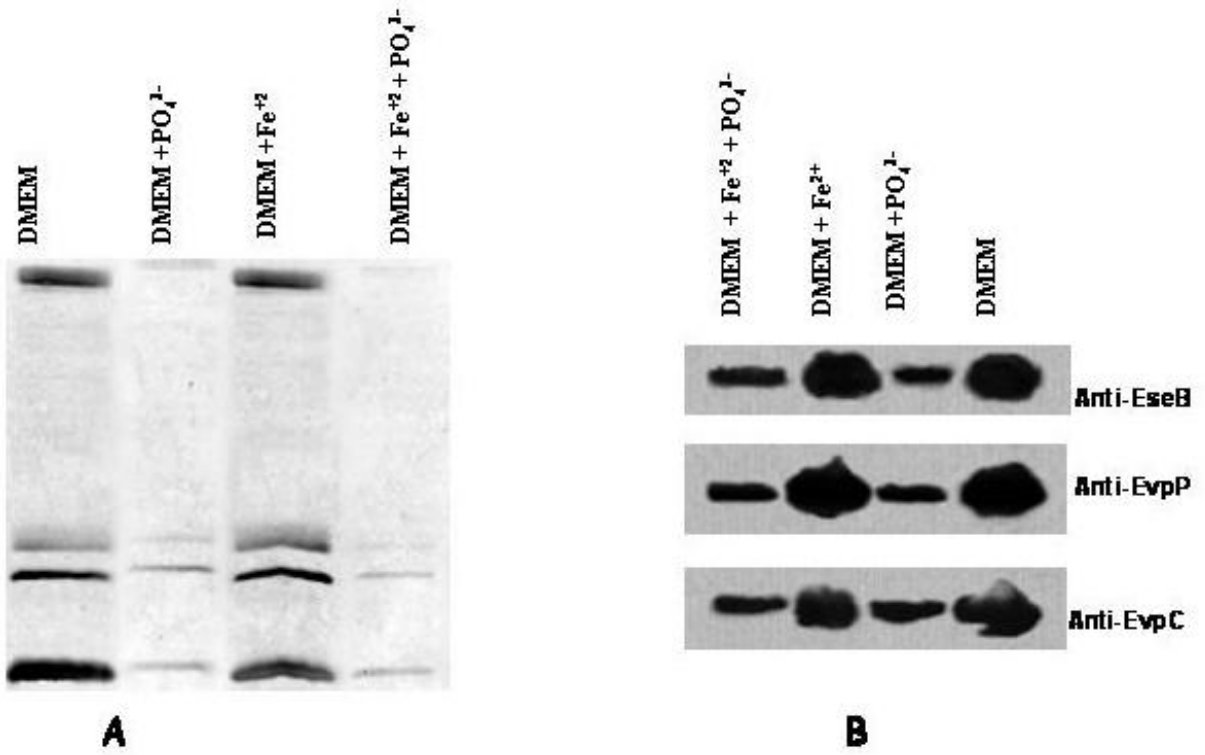


Fig. IV.16. Additive effect of phosphate and iron on the expression and secretion of T3SS and T6SS proteins in *E. tarda Δfur*. Silver stained SDS PAGE showing T3SS and T6SS ECPs secretion (A) and (B) Western blot analysis showing the expression of EseB from T3SS; EvpP and EvpC from T6SS by *E. tarda Δfur* cultured in DMEM in presence or absence of phosphate and iron as indicated. Both the ECP and TCP fractions were adjusted for equal number of bacterial cells as determined by standard plate count.

sequence (Fur box) mapped on the promoter region of a targeted gene and inhibits the transcription of the gene. Fur box is identified upstream of *evpP* which is also regulated by iron and EsrB. Non-polar deletion mutant of *fur* (*Δfur*) was created to identify the role of Fur in response to *E. tarda* virulence. The growth and protein secretion profiles of the wild type PPD130/91 and *Δfur* were measured at 30^{o}C in presence or absence of phosphate and/or iron. SDS-PAGE analysis showed that *Δfur* mutant was prolific in the secretion and expression T3SS and T6SS proteins in absence of both iron and phosphate (ECP=5.75 ± 0.15 μg/ml, *n*=3). *E. tarda Δfur* mutant secreted 70% more ECP in comparison to the wild type bacteria (Fig. IV.16). Mutant *Δfur* shows 75% increase in the expression of *esrC*-LacZ in comparison to the wild type (Fig. IV.17) In contrast, expression of *esrB*-LacZ in *phoUi* remains at similar level as wild type bacteria (Fig. IV.17). Similar to PhoU, our results suggest that Fur exert its function through modulation of *esrC* but not *esrB*. Higher secretion of type III and typeVI proteins by the mutant suggests negative regulatory activity of Fur. Interestingly *Δfur* could no longer respond to iron suggesting its possible role as an iron sensor. Although *Δfur* could not respond to iron changes but it still responds to phosphate changes suggesting a cross-talk between *pstSCAB-phoU* and *fur*.

IV.4 Discussion:

This study has established how environmental factors such as phosphate and iron can regulate the expression and secretion of virulence proteins for the bacteria to invade inside host tissues. In *E. tarda*, a transposon mutant (306) which has an insertion in a putative membrane protein was shown to affect the expression of the T3SS. Unlike OmpR-EnvZ in *Salmonella* species, this mutant did not affect the expressions of *esrA* or *esrB* but suppressed the expression of *esrC*

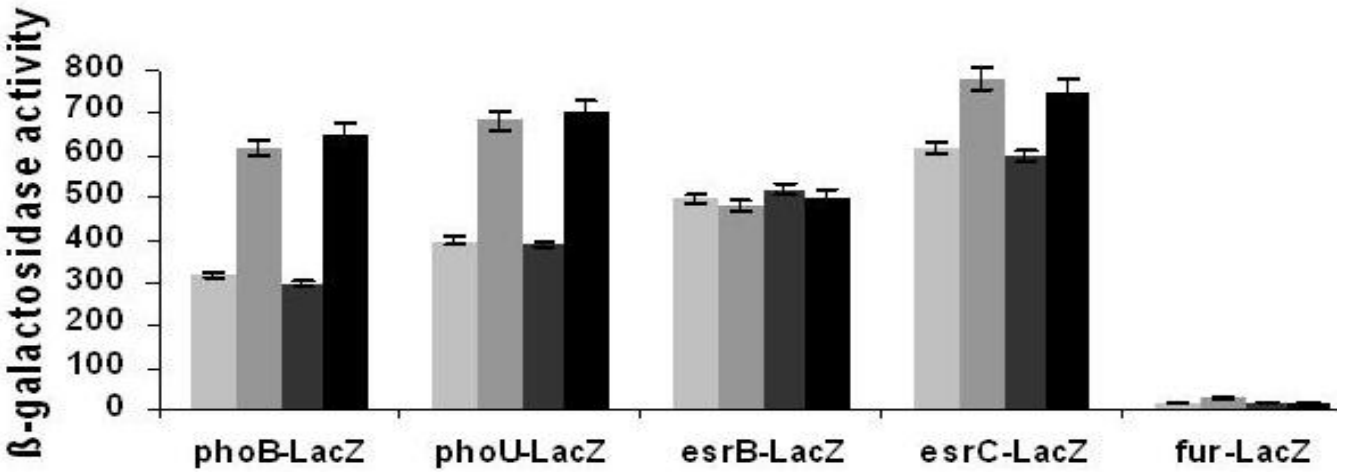


Fig. IV.17. Effect of phosphate and iron on the expression of *phoB*, *phoU*, *esrB*, *esrC* and *fur* in *E. tarda Δfur*. The ß-galactosidase activities of the reporter genes *phoB*-LacZ, *phoU*-LacZ, *esrB*-LacZ and *esrC*-LacZ were determined in *E. tarda Δfur* in DMEM in presence or absence of iron and/or phosphate as indicated. The values represent the mean ± SD from one representative experiment performed with triplicate samples. Equivalent results were obtained at least in triplicates.

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and the T3SS apparatus gene *esaC*. Interestingly, transposon insertion in *pstC* also has a similar effect which inhibited the expression of *esrC*, suggesting that the PST operon has some control over the expressions of type III and type VI gene clusters. (Zheng et al, 2005a). Similarly, in this study we have seen that *phoB/phoR* as well as *pstSCAB-phoU* works through the modulation of *esrC* but not *esrB*. Self-regulatory PhoB binds to the polycistronic promoter of pstSCAB-phoU and positively regulates the transcription of genes involved in high affinity phosphate transporter system. Inspection of the type III and type VI gene clusters showed presence of a putative pho box in the promoter region of evpA, which codes for a non-characterized bacterial T6SS protein. Both iron and phosphate negatively affects the secretion as well as expression of T3SS and T6SS proteins in an additive manner in which the effect of iron is more significant than that of phosphate. Fe^{2+} and phosphate exert an opposite effect on the expression of genes belonging to two different regulatory cascades namely *fur* and *pstSCAB-phoU* suggesting a negative cross-talk between them. In *E. coli*, PhoU had no effect on the uptake of Pi and *phoU* mutant showed sever growth defect. In *E. tarda* PPD 130/91, PhoU behaves as an activator and it regulates both the expression and secretion of T3SS and T6SS proteins by modulating the expression of *esrC*. PhoB is positively regulated by PhoU and the effect of phosphate on the expression of *phoB* is *phoU* dependent. Fe^{2+} can still activate the expression of *phoB* in the absence of *phoU* confirming the additive effect of iron and phosphate. Similar to PhoB, PhoU has been shown to exert a suppressing effect on the expression of *fur* in a phosphate dependent manner. Fur is identified as a suppressor of T3SS and T6SS proteins by regulating the expression of *esrC*. Fur behaves as a Fe^{2+} sensor and inhibits the expressing levels of *phoB*, *pstS*, and *phoU* genes which are responsible for high affinity phosphate uptake.

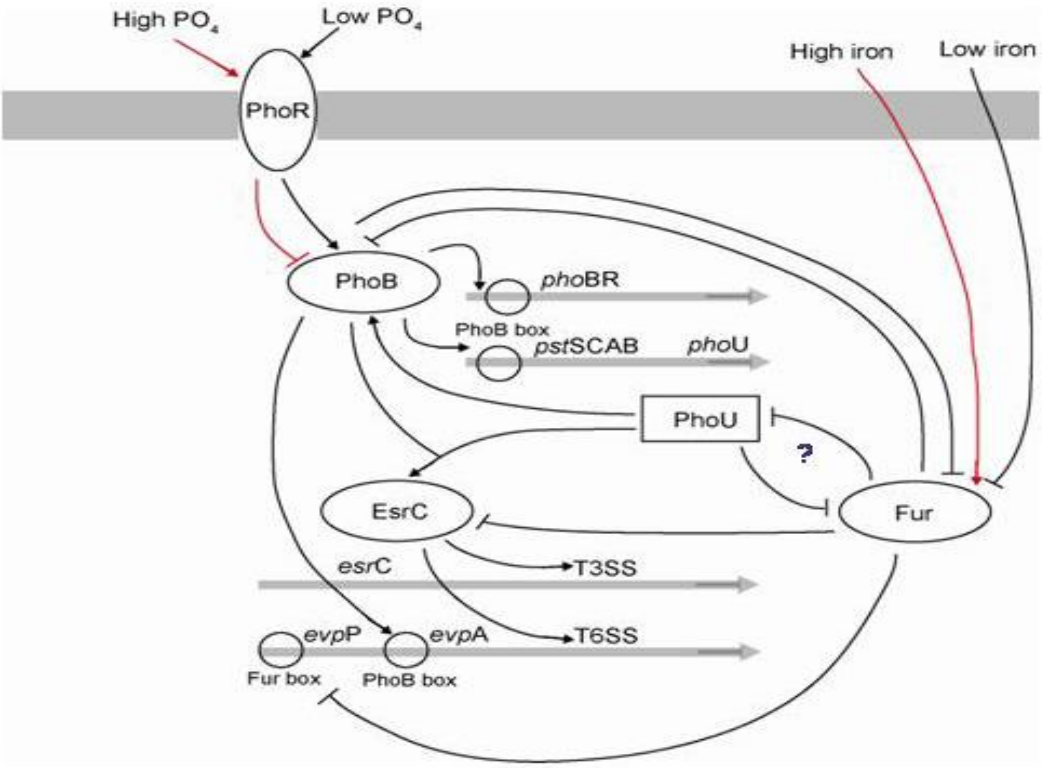

Fig. IV.18. Model illustrating the phosphate and iron regulation of T3SS and T6SS by the PhoB-PhoR and Fur system

Our results shown that inactivation of *pstSCAB-phoU* operon enhanced the transcription of *fur* which in turn suppressed the secretion of T3SS and T6SS proteins making *E. tarda* highly avirulent. In *E. coli*, the transposon inserted at the *pstC* gene had pleiotropic effect which not only affected the Pi transport but also reduced bactericidal resistance to serum, production of polysaccharides and mutant carrying the transposon became avirulent in pigs (Daigle et al, 1995). In *Streptomyces lividans*, mutants lacking *phoP* and both *phoPR* were unable to grow at low phosphate conditions and the production of extra-cellular alkaline phosphate was also hindered suggesting PhoP/R involvement in alkaline phosphate regulation. Many secondary metabolites like siderophores, antibiotics, and pigments were up regulated at low phosphate conditions which may suggest that PhoP/R play an important role in many cellular functioning (Sola-Landa et al, 2003).Disruption of *fur led* to the induction of *pstSCAB-phoU* operon which acts as positive regulators and coordinate with *esrC* in regulating the T3SS and T6SS proteins. Systemic control of regulation cascade is important for maintaining homeostasis in bacteria. *E. tarda* infects many different fish species, such as blue gourami fish and channel catfish. During edwardsiellosis, high content of organic material in surrounding water leads to high iron and high phosphate conditions inducing the activity of negative regulator Fur. Fur in turn preserves the energy in the bacteria by suppressing the secretion of virulence proteins to the outside environment. Upon being engulfed by fish, bacteria sense rapid decrease of iron and phosphate concentrations and recognize the presence of host. At the outset, low iron and low phosphate triggers the positive activators PhoB and PstSCAB-phoU and at the same time suppresses the negative regulator Fur. As a result, the bacteria become highly secretion proficient and can exploit the host immune responses by releasing the virulence proteins ensuring its survival.

Chapter V. General conclusion and Future direction

V.1 General Conclusion

Cell-to-cell communication in bacteria is an intricate process that relies on the detection and response to enviornmental stimuli as present within the host body. Often, bacteria use multiple regulatory cascades to obtain information and respond to the environmental stimuli. In this study we have used both genomics and proteomics approaches to show the regulation of *E. tarda* in response to various environmental conditions such as temperature, Mg^{2+}, antimicrobial peptides, pH, phosphate and Fe^{2+}. *E. tarda* shows maximum secretion of virulence proteins from 23°C to 35°C with severe secretion defect at or below 20°C and at or above 37°C which represents two distinct non-virulent states of the bacteria. Low Mg^{2+} concentration induces the secretion of virulence proteins and acts with temperature in an additive manner. We have identified PhoQ as the first reported temperature sensor in *E. tarda* showing significant change of secondary structure with a temperature shift from 30°C to 37°C. PhoP, by binding to the promoter region of *esrB*, positively regulates the type III and type VI secretion system and the secretion defect at 37°C can be justified from the conformational change of PhoQ sensor domain at 37°C. We have identified specific Pro and Thr residues in the PhoQ sensor domain of *E. tarda* which are responsible for its low melting point. By changing specific amino acid residues to the corresponding residues of *S. typhimurium*, we identified Pro 120 and Thr 167 as the key residues responsible for the lower temperature stability of *E. tarda* PhoQ. Complementation of $phoQ_i$ with the thermally stable mutants, resulted in a 'loss of function' phenotype except for P77L, which showed constitutive secretion even at 20°C resembling a temperature blind condition at 20°C. We also identified acidic cluster residues on PhoQ as the Mg^{2+} binding site which can stabilize the sensor domain of PhoQ against urea and themal

denaturation. We also showed that PhoQ of *E. tarda* is also responsive to acidic pH and anti-microbial peptides which act additvely regulating the secretion of T3SS and T6SS proteins. Based on the TCP profile, 20°C and 37°C represents two distinct states of the bacteria where at 20°C, *E. tarda* attained a rod-shaped conformation which changed to a coccoid form at 37°C. At 35°C, *E. tarda* exhibits an intermediate cocco-bacllius phenotype. We extended our regulation study by investigating two more environmental factors, phosphate and iron, on the virulence of *E. tarda*. Addition of phosphate and iron highly suppressed the expression and secretion of type III and type VI proteins in an additive manner. We sequenced the two-component regulatory system PhoB/PhoR and showed binding of PhoB to the polycistronic promoter and positively regulating high affinity phosphate transport system *pstSCAB-phoU*. PhoB also regulates the expression of a T6SS protein, EvpC, by binding to the promoter region of *evpA* present upstream of *evpC*. Fur is an iron sensor which acts as a negative regulator by supressing the activity of *esrC*. Addition of iron activates transcription of *fur* but supresses the expression of *pstSCAB-phoU* and *phoB*. Similarly, addition of phosphate suppresses the transcriptional activities of *pstSCAB-phoU* and *phoB* butactivated the transcription of *fur*. We propose a negative cross-talk between the phosphate transport system PstSCAB-phoU and the iron induced Fur. Our findings support the theory in which *E. tarda* face low phosphate and Fe^{2+} concentrations within the phagosomes inside the host cells enabling it to exert maximim virulence to survive against host immune responses. Outside the host body, bacteria preserve energy and reside in a non-virulent state. In this study we have documented enviornmantal signals from the host which induces the bacteria to become highly lethal and strengthen its armor against host responses. More work is required to identify and relate new proteins and signals which are involved in the recognition and cross-talk between pathogens and their hosts.

This kind of studies can also be extended to other gram negative pathogenic bacteria to identify the differences in the phenotype between species arising through horizontal gene transfer. Therefore our future work will focus on the followings:

V.2 Future direction

1. To identify and characterize the functions of protein encoded in the *pstSCAB-phoU* operon by systematical deletion of individual genes. This will give important informations about the genes and their roles in the secretion of type III and type VI proteins.

2. To study the effect of phosphate and iron on the secretion of virulence proteins in EPEC. This will facilitate understanding the differences between species belonging to the same genus.

3. To map the binding domain of EsrB and EsrC within the type III and type VI gene clusters. As the transcriptional activities of *esrB* and *esrC* vary in response to different environment signals, it will be useful to identify the exact binding sites of both the regulatory proteins to understand gene specific enviornmental responses.

4. To study the presence of other genes containing the PhoP box, Fur box and Pho box in their promoter regions which can help in the identification of new proteins involved in

the cross-regulation cascades. This will fish out novel transcriptional regulators that respond to specific environmental cues.

5. To study the effect of phosphate and iron on the transcription of specific genes present within the type III and type VI clusters based on the EsrB and EsrC binding map.

6. To study the effect of various enviornmental signals on the TCP profile of non-polar deletion mutants of *esrB* and *esrC* in a concentration dependent manner.

7. To identify the proteins involved in the morphological changes in *E. tarda* in response to temperature. As *E. tarda* shows drastic changes in phenotype at two extreme temperatures, two-diemensional PAGE followed by mass spectrometry can be used to identify proteins expressed by bacteria under different incubation conditions. Deletion mutants can be generated to identify proteins rendering morphological changes in *E. tarda*.

8. To analyze the negative cross-talk between PstSCAB-phoU, PhoB/PhoR and Fur regulon. GST-pull down assay and yeast two hybrid system can be used to identify possible interactions between the regulatory proteins.

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