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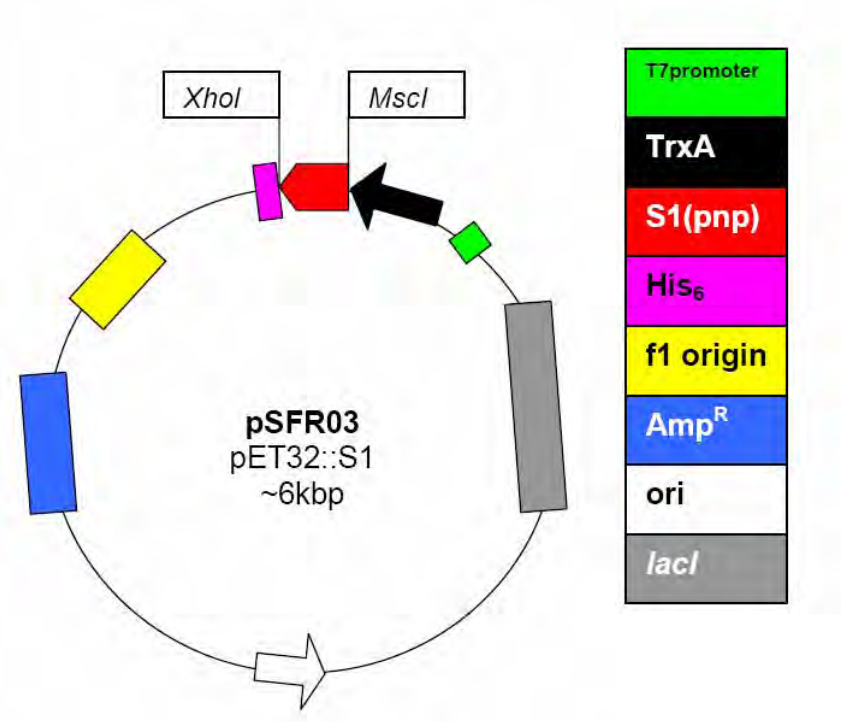
Dissecting the role of polynucleotide phosphorylase in virulence gene expression in *Salmonella enterica*



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SUMMARY

The gene *pnp* that codes for the exoribonuclease polynucleotide phosphorylase (PNPase) is found omnipresent in bacteria, plants and animals. PNPase was also found to have diversified influences in different life forms such as growth at low temperature, virulence etc. Structurally, PNPase has five distinct domains, where the S1 domain acts as RNA binding domain. *Salmonella enterica* (*S. enterica*) responsible for both acute and persistent infections in human, serves as a good study model for bacterial virulence. PNPase of *S. enterica* was reported to have effects on persistency and virulence.

This project was focused to assess the effects of different parts of PNPase, especially regarding its S1 domain, on the virulence of *S. enterica*. To do so, I constructed recombinant plasmids containing the s1 domain of *pnp* (pSFR01, pSFR02 and pSFR03). An N-terminal thioredoxin fusion was found necessary for the expression of the recombinant S1 protein. With the plasmid pSFR03 (having N terminal thioredoxin and C terminal poly histidine tag), recombinant S1 protein was successfully expressed, detected and purified. To investigate S1 domain effects, plasmid constructs having full length or truncated forms *pnp* were tested against *pnp* proficient and *pnp* deficient background in *Escherichia coli* and *S. enterica*. Through a β-galactosidase assay, it was shown that the s1 domain of *pnp* itself could down-regulate the expression of *Salmonella* plasmid virulence (*spv*) genes in *pnp*-deficient *S. enterica* under *in vitro* growth conditions stimulating *spv* expression. It was also found that, besides S1 domain, other regulators might also be involved for *spv* gene expression in *S. enterica*.

INTRODUCTION

Polynucleotide Phosporylase

Though it was discovered back in 1955 by Grunberg-Manago and Ochoa[12], polynucleotide phosphorylase [PNPase, polyribonucleotide:orthophosphate nucleotidyltransferase (EC 2.7.7.8); encoded by the *pnp* gene] is still at the center of interest in biological research. This is because *pnp* is ubiquitous not only in different forms of life (bacteria[1], plants[20], animals[23] including human[22]) but also has significant effects on a variety of organisms for e.g. growth of bacteria at lower temperature [48, 27] or polyadenylation in plants[2]. Nevertheless, when it comes to gene regulation, the knowledge of structure-function relationships with respect to this complex enzyme is still largely incomplete.

The enzymes that control the stability and decay of RNAs are defined as ribonucleases [8]. These enzymes act on RNA either at the terminals (exoribonucleases) or within (endoribonuclease) [7]. In *Escherichia coli* (*E. coli*), there are eight exoribonucleases [36]. Out of these, PNPase and ribonuclease PH (RNase PH) degrade RNA through a phosphate-dependent 3′ to 5′ phosphorolysis reaction [25]. PNPase also can polymerize RNA (5′ to 3′) under conditions of low inorganic phosphate (Pi) [11, 26, 30, 31]. Interestingly, PNPase was initially reported as the first enzyme able to synthesize polynucleotides from ribonucleotides, without requiring a template[12]. PNPase plays the central role in polyadenylation in spinach chloroplasts, cyanobacteria and Gram-positive bacteria [2]. In humans, most of the PNPase is located in the mitochondrial intermembrane space, may not play the major role in the processing and degradation of RNA [4].

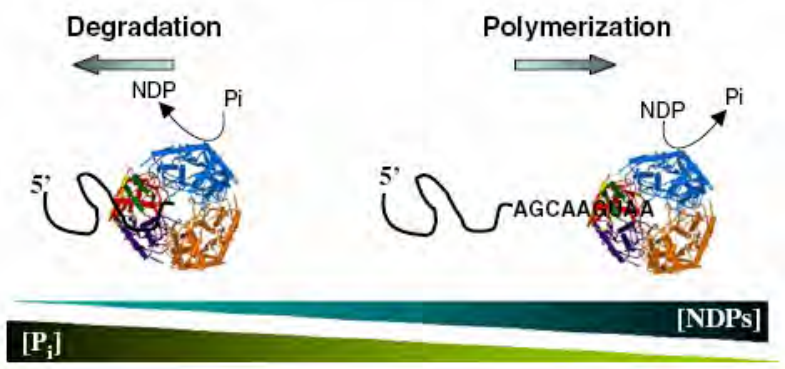

Fig. 1 A model for possible modulation of enzymatic activities of PNPase as an exoribonuclease or a polymerase.[Courtesy Schuster *et al* (2007) [24] –an open choice article, use for any non commercial purpose is permitted by publisher (www.springerlink.com)]

Besides acting as a ribonuclease, PNPase also influences bacterial growth and virulence, i.e. the relative ability of a microbe to cause disease. It is vital for the growth of *E. coli*, *Bacillus subtilis* and *Yersinia enterocolitica* at low temperature [48, 27]. In *Salmonella enterica* (*S. enterica*), PNPase controls the pathogenesis and persistence in a universal manner [5, 47]. In *Yersinia pseudotuberculosis,* a close relative of *Yersinia enterocolitica* (a rod shaped bacterium commonly cause diarrhea), PNPase finely tunes the *Yersinia* type three secretion system (T3SS) [39]. Type three secretion systems are supramolecular organelles that inject

virulence-associated effector proteins directly into host cells [39]. Also in *Salmonella*, the virulence is dependent on T3SS and regulated by PNPase [5].

The *pnp* gene from *E. coli* was the first one sequenced [35]. Structurally it has five domains [1]. These are two ribonuclease PH (RPH)-like (pnp1 and pnp2) domains, one alpha helical, one KH, and one S1 domain [1]. The crystal structure of the *Streptomyces antibioticus* PNPase showed it as a doughnut-shaped trimeric protein [1]. The monomers form a central channel which is the predicted site for catalysis [1]. On top of the enzyme, the KH and S1 RNA binding domains are placed [43]. The KH domain lies just above the central channel and while the S1 domain faces outward [43]. The S1 domain was initially identified in ribosomal protein S1[1]. It is also found in other RNA-associated proteins for e.g. bacterial translation initiation factor 1 (IF1), eukaryotic translation initiation factor (eLF2), ribonuclease II, ribonuclease E and so on [3]. It is also reported that the domains are well conserved among bacteria [1].

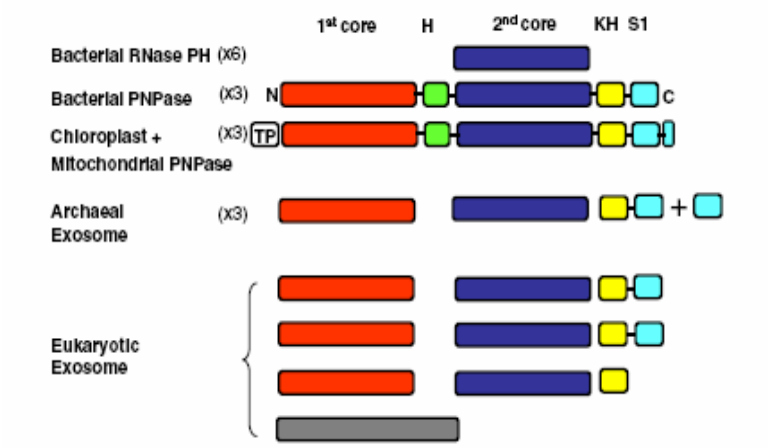

Fig. 2 Different domains present in the RNase PH, PNPase and exosome (-a macromolecular complex involved in RNA degradation). The two core RNase PH domains of PNPase are shown in red and dark blue and the KH and S1 domains are shown in yellow and pale blue, respectively. The exosome protein related to the bacterial RNase II is shown in gray. The number of monomeric units needed a make the functional complex for each enzyme is indicated by the (x3 or x6) [Courtesy of Lin-chao *et al* (2007)[24] –an open choice article, use for any non commercial purpose is permitted by publisher (www.springerlink.com)]

PNPase controls its own expression [37]. Ribonuclease III initiates cleavage at the 5′ end of PNPase messenger RNA, followed by 3′–5′ degradation by PNPase [17]. For this auto-control, both the enzyme activity and the presence of the RNA binding domains are necessary [16]. Mutations, especially in the KH and S1 domains may hamper the growth of *E. coli* at low temperatures [28]. It was reported that, only the S1-domain can restore the *Yersinia* T3SS activity in a *pnp* mutant [38].

Salmonella - a model for bacterial virulence study

Salmonella is a genus of gram-negative rod shaped bacteria that can cause both acute and persistent infections [14]. They are named after the scientist who discovered them, Dr. Daniel Salmon [40]. The genus has two species *Salmonella bongori* (*S. bongori*) *and Salmonella enterica* (*S. enterica*) [40]. *S. enterica* is a facultative intracellular pathogen- able to grow either inside or outside a host [40]. On the basis of cell surface structure; there are over 2500

known types, or serotypes of *S. enterica* today [46]. Acquisition of a number of genetic elements such as the *Salmonella* pathogenicity islands (SPIs), the *Salmonella* plasmid virulence (*spv)* gene cluster and selected prophages through the evolution may have caused the emergence of these numerous serovars of *Salmonella* [47]. The SPIs are distinct parts of genome acquired by horizontal (cell to cell, not offspring) transfer and codes for T3SS. All of these genetic elements improve different virulent properties like bacterial invasion, pro-inflammatory responses, intracellular survival and replication [47].

According to the World Health Organization (WHO), salmonellosis is generally defined as disease caused by the genera *Salmonella* (that also includes serovars Typhi or Paratyphi) [46]. Most salmonellosis are common and widely distributed food-borne diseases constituting a major public health burden and representing a significant cost in many countries [46]. Millions of human cases are reported worldwide every year and salmonellosis results in hundreds of thousands of deaths [46]. Typhoid fever is a typical example of severe systemic salmonellosis or *Salmonella* infection in humans [5, 34]. It is involved in an estimated 16.6 million new cases per year [34]. Of the convalescents, up to 5% develop into chronic carrier state [9]. *S. enterica* serovar Typhimurium (*S*. Typhimurium) can cause an invasive infection in mice that is similar to the acute phase caused by *S.* Typhi in human [42]. Therefore, the murine (mice) salmonellosis is a classic experimental model for studying systemic salmonellosis [42]. In the *S*. Typhimurium *pnp* gene, Clements *et al* (2002) [5] identified a single point mutation that affected *Salmonella* pathogenicity island 1(SPI-1)-mediated invasion and *spv* gene cluster and *Salmonella* pathogenicity island 2 (SPI-2)-mediated intracellular replication and the mutant also able to cause persistent infection. Using microarray analysis, they also reported that PNPase affected the mRNA levels of a subset of virulence genes especially those in SPI-1 and SPI-2 [5]. Their results suggested a connection between PNPase and *Salmonella* pathogenesis and that these modifications in the activity of PNPase affected the persistency of *Salmonella* [5]. In 2006, Ygberg *et al*. [47] reported that the expression of the virulence-associated *spv* gene cluster was increased when a PNPase-deficient mutant was grown under *in vitro* conditions those mimicked the *in vivo* condition that stimulates the infection inside the host. This clearly indicated that PNPase acted as a suppressor of *spv* gene expression [47].

Transcription of the *spv* gene cluster is regulated by two main promoters [6]. One is in front of the transcriptional activator protein gene *spvR* (P*spvR*) [6, 19, 44]. The other lies in front of *spvA* (P*spvA*) directing the of Spv effector proteins expression [6, 19, 44]. Helskanen *et al.* (1994) [14] constructed a recombinant plasmid pHUB61with a *lacZ* reporter gene that (encoding β-galactosidase) fused to the *spvA* gene of *S.* Typhimurium, this can be used as a tool to analyze the association between PNPase and *spvA* hence *spv*.

Constructing recombinant plasmid- the general approach

To construct a recombinant plasmid for the expression of a protein, it is important to select and clone the gene(s) of interest in an expression vector and to use an appropriate expression system. The pET system is widely used system developed for the cloning and expression of recombinant proteins in *E. coli.* Originally developed by Studier and colleagues, in pET vectors, target genes are cloned under the control of strong bacteriophage T7 transcription and translation signals [29, 41]. The expression is induced by providing a source of T7 RNA polymerase in the host cell [29]. To speed up the protein production, detection and purification, the strategy of parallel expression of a protein from a variety of vectors containing different

tags and/or fusion partners (for e.g. poly-histidine or thioredoxin), and different of *E. coli* host strains are commonly adopted [10].

The β-galactosidase activity test

To study the transcriptional regulation of genes, assaying the β-galactosidase activity is widely used [49]. The β-galactosidase activity can be assayed by determing the hydrolysis of the chromogenic substrate, o-nitrophenyl-β-D-galactoside (ONPG) [49] as follows;

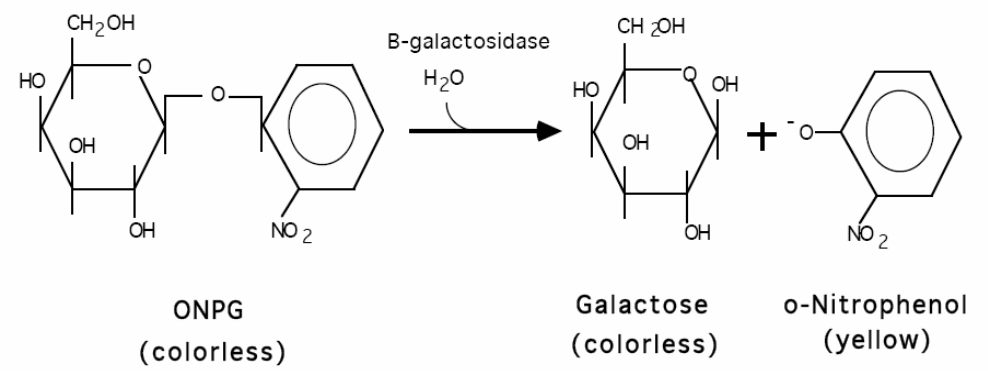

Fig. 3 Chemical reaction of β-galactosidase assay

According to Zubay *et al.*(1972) [49] the amount of o-nitrophenol formed can be measured by determining the absorbance at 420 nm. The reaction is stopped by addition of Na_2CO_3 which increases the reaction pH to 11. At this pH most of the o-nitrophenol is converted to the yellow colored anionic form and β-galactosidase becomes inactivated. To run the reaction, whole cells are permeabilized to allow ONPG to enter the cytoplasm. However, since whole cells are present, the absorbance at 420 nm is the sum of the absorbance due to o-nitrophenol and light scattering due to the cells. The contribution of light scattering can be determined by measuring the absorbance (optical density, OD) at 550 nm where o-nitrophenol doesn't absorb. The light scattering at 420 nm is 1.75x the light scattering at 550 nm, so the absorbance of onitrophenol is determined by subtracting 1.75 x OD_{550} nm. The corrected absorbance is then used to calculate the activity of β-galactosidase [49].

AIM

The aim of this project was to investigate the influence of the PNPase- S1 domain on *Salmonella*'s virulence. To do so, the very first step was to construct a functional recombinant S1 domain plasmid. Here I report successful construction, expression of recombinant S1 domain plasmid(s) and purification of its product together with its primary influence on *Salmonella* virulence gene *spv*.

RESULTS

Construction of recombinant plasmids

To construct a functional recombinant S1 domain plasmid, primers were designed with corresponding restriction sites were used to amplify the s1 domain region of the *pnp* gene from the *S.* Typhimurium MC1 strain's genomic DNA through polymerase chain reaction (PCR). The PCR products were analyzed through agarose gel electrophoresis. Figure 4 shows the agarose gel electrophoresis of the PCR-amplified s1 region of *pnp* of *S.* Typhimurium MC1 strain.

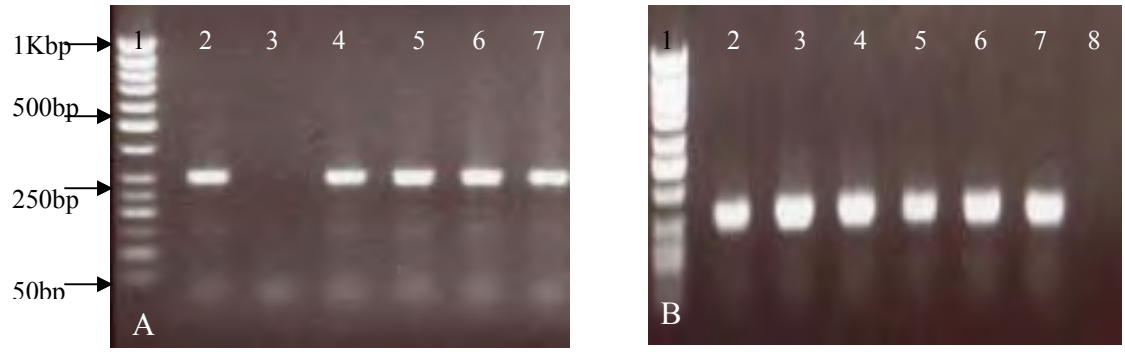

Fig. 4 Agarose gel electrophoresis of PCR amplicons from the s1 region of the *S.* Typhimurium (MC1) *pnp* gene. The amplicons were generated by PCR using Set 1 (**A**) and Set 2 (**B**) primers and MC1 genomic DNA as template. **A.** In the gel, the DNA marker (50 bp ladder) is in lane 1, lane 2 and 4-7 show the 295bp amplicon bands and the lane 3 shows the negative control. **B.** In this gel, the DNA marker (50 bp ladder) is in lane 1, lane 2 to 7 shows the 316bp amplicon bands and the lane 8 shows the negative control.

In order to insert the PCR-amplified s1 region into the plasmid vector, both the PCR products and the vector plasmids (pET32a, pET22b, Appendix I) were cleaved by the corresponding restriction endonuclease enzymes. Figure 5 shows the agarose gel electrophoresis of restriction endonuclease-cleavaged recombinant plasmids and PCR products.

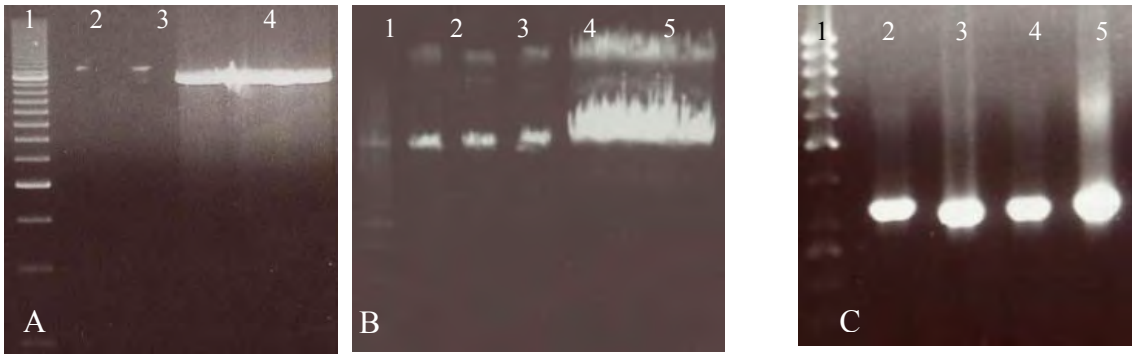

Fig. 5 Agarose gel electrophoresis of restriction cleavage patterns of plasmid vectors and PCR amplicons. **A**. In this gel, the lane 1 shows the DNA marker (500bp ladder), lane 3 shows uncut pET 32a(+) vector and lane 4 shows the *Msc*I and *Xho*I double restriction cut of pET 32a(+).**B.** In this gel, the lane 1 shows the DNA marker (500bp ladder), lane 2 shows the pET 22(b) vector with *Xba*I single cut, lane 3 shows pET 22(b) vector with *Xho*I single cut, uncut pET 22(b) in lane 4 and pET 22(b) with *Xba*I and *Xho*I double cut in lane 5. **C.** In this gel, lane 1 the DNA marker (50bp ladder), lane 2 shows the uncut PCR amplicon (with Set 1 primers), lane 3 shows PCR amplicon (with Set 1 primers) double cut with *Msc*I and *Xho*I enzymes, lane 4 shows PCR amplicon (with Set 2 primers) double cut with *Xba*I and *Xho*I and lane 5 shows uncut PCR amplicon (with Set 2 primers).

The restriction products from the vector and PCR amplicons were extracted from the agarose gel and ligated to generate the recombinant S1 domain plasmid (pSFR01 and pSFR02). Figure 6 shows the agarose gel analysis of the ligated plasmid with insert and plasmid vector.

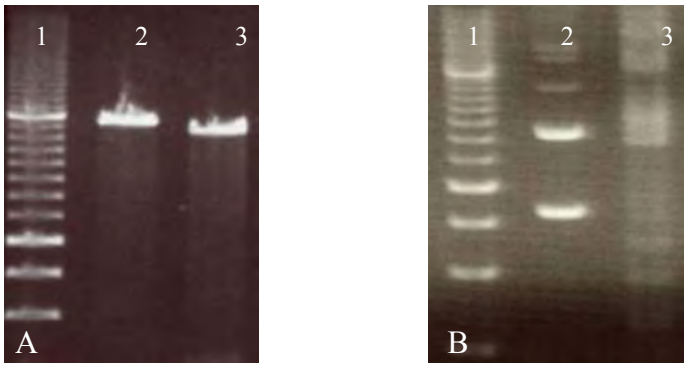

Fig. 6 Agarose gel electrophoresis of the ligation reaction. A. In this gel, lane 1 shows the DNA marker (500bp ladder), lane 2 shows the s1 region of *pnp* insert-ligated pET 32a(+) plasmid (pSFR02) and lane 3 shows the pET 32a(+) plasmid vector. **B.** In this gel lane 1 shows the DNA marker (500bp ladder), lane 2 shows pET22(b) plasmid vector and lane 3 shows s1 region of *pnp* insert ligated pET 22(b) plasmid (pSFR01).

To generate the recombinant plasmid encoding an N-terminal Trx-tagged and a C-terminal His_6-tagged s1 domain, the ligated plasmids (pSFR01 and pSFR02) were cleaved in with restriction endonuclease *Pst*I. *Pst*I cuts at codon 666-667 of *pnp* in the s1 domain coding sequence and in the beta lactamase coding sequence of pSFR01 (4760 in pET32a) and pSFR02 (4353 in pET22b). The reaction mixtures with each plasmid were then run in 0.8 % agarose in gel. The higher molecular weight (~4.6kbp) band of pSFR01 (having part of amp-trx-part of s1 sequence) and the lower molecular weight (~1.3kbp) band of pSFR02 (having the rest of the s1 sequence-his_6(3′)-amp sequence) were extracted from the gel and ligated together, generating the recombinant plasmid pSFR03, which encodes the Trx -S1 region-His_6 recombinant protein (Figure 7).

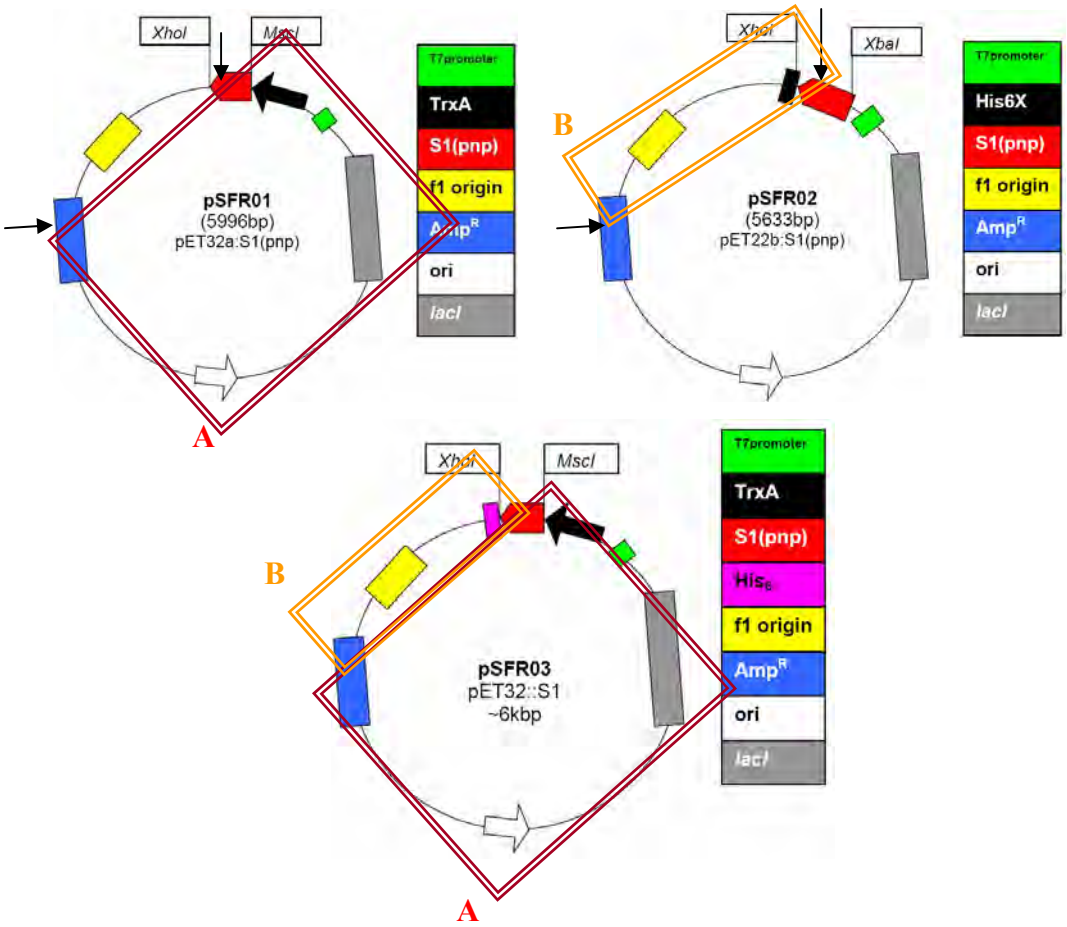

Fig. 7 Construction of the recombinant plasmid pSFR03. The black arrows pointing at the *Pst*I sites in pSFR01 and pSFR02. The box A shows the part of pSFR01 and the box B shows the part of pSFR02- those were ligated together to generate pSFR03.

To check whether the desired gene fragment was inserted in plasmid constructs, PCR and restriction endonuclease cleavage were carried out with respective primers and restriction enzymes. Figure 8 shows the agarose gel analysis of cloned insert in plasmid constructs as confirmed by PCR while figure 9 shows the agarose gel analysis of restriction endonuclease-cleaved recombinant plasmids.

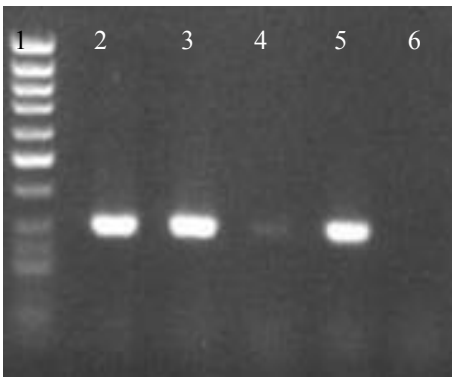

Fig. 8 Agarose gel electrophoresis of the PCR amplicons (the s1 region of *S.* Typhimurium *pnp*) from the plasmid constructs. Lane 1 shows the DNA marker (50 bp ladder), lane 2 shows the PCR amplicon with pSFR01 template, lane 3 shows the PCR amplicon with pSFR02 template, lane 4 shows the PCR amplicon with *E. coli* BL21 genomic DNA as template (positive control), lane 5 shows the PCR amplicon using pSFR03 as template and lane 6 shows the PCR amplicon from *E. coli* BL21 (Δ*pnp*) genomic DNA (negative control).

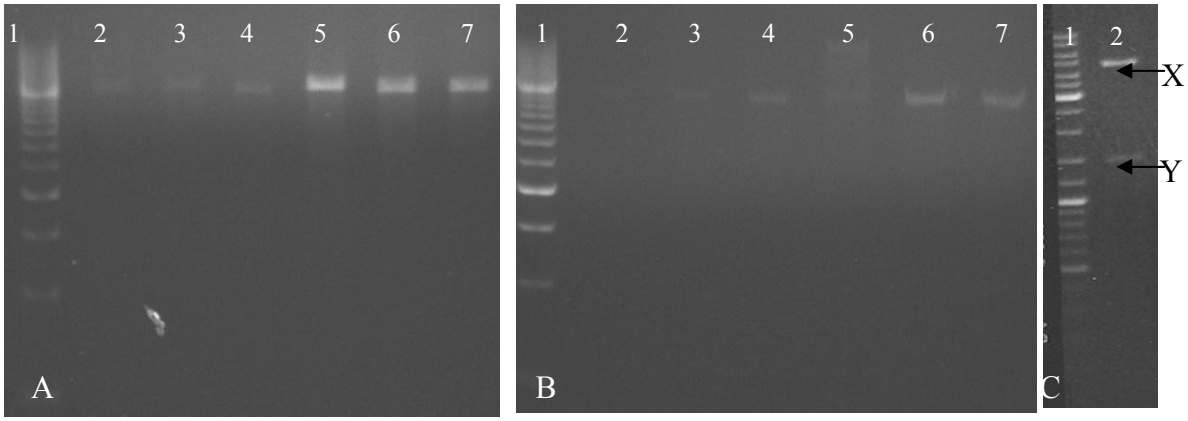

Fig. 9 A. Agarose gel electrophoresis of the restriction endonuclease cleavage reaction. A. In this gel, lane 1 shows the DNA marker (500bp ladder), lane 2 shows pSFR01 cut with *Msc*I, lane 2 shows pSFR01 cut with *Xho*I, lane 4 shows pSFR01 double cut with *Msc*I and *Xho*I, lane 5 shows pET 32a (+) cut with *Msc*I, lane 6 shows pET 32a (+) cut with *Xho*I, lane 7 shows pET 32a (+) double cut with *Msc*I and *Xho*I. **B**. In this gel, lane 1 shows the DNA marker (500bp ladder), lane 2 shows pSFR02 cut with *Xba*I, lane 2 shows pSFR02 cut with *Xho*I, lane 4 shows pSFR02 double cut with *Xba*I and *Xho*I, lane 5 shows pET 22(b) cut with *Xba*I, lane 6 shows pET 22(b) cut with *Xho*I, lane 7 shows pET 22(b) double cut with *Xba*I and *Xho*I. **C.** In this gel, lane 1 shows the DNA marker (GeneRuler DNA ladder mix), lane 2 shows restriction cleaved pSFR03 X shows the ~4.5kbp band originally a part of pSFR01and Y shows the ~1.5kbp band originally a part of pSFR02.

Figure 10 shows maps of the recombinant plasmids (pSFR01, pSFR02, pSFR03) constructed in this study.

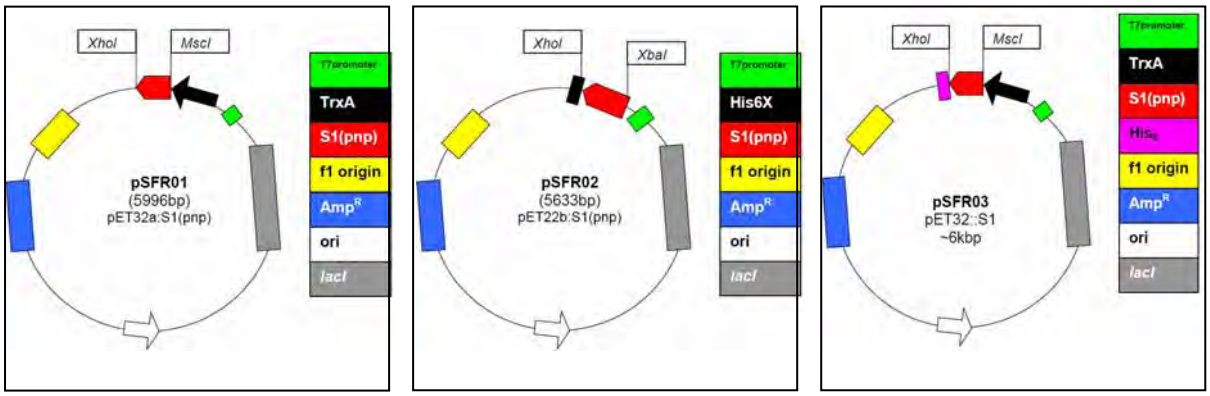

Fig. 10 Map of the plasmid constructs

Protein expression analysis

To check the protein expression of the plasmid constructs, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out. Overexpressed proteins (induced by 1 mM IPTG) were found as abundant bands after staining with Coomassie blue. Figure 11 shows SDS-PAGE analysis of prtoein extrafts obtained from *E. coli* BL21 (*Δpnp*) containing different plasmid constructs.

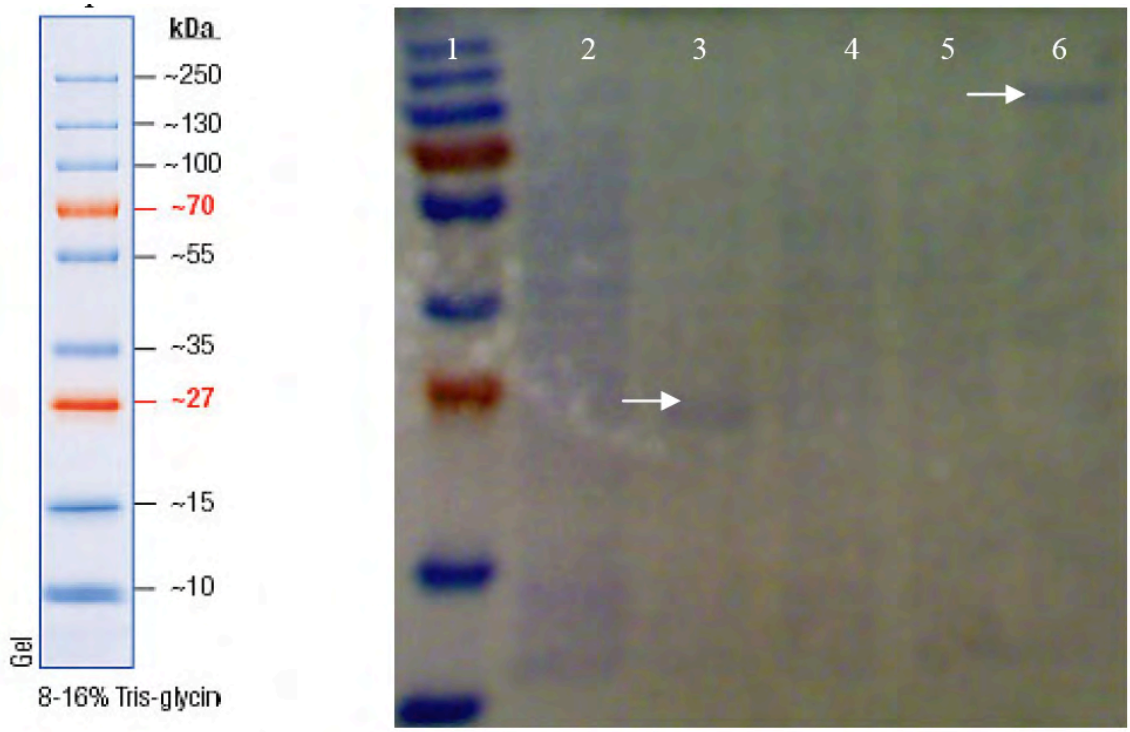

Fig. 11 SDS PAGE analysis of the protein expression by the plasmid constructs. In this gel, lane 1 shows the protein marker (PageRuler from Fermentas), lane 2 shows protein profile of BL21 (Δ*pnp*) with pET32a (vector control), lane 3 shows the protein profile of BL21 (Δ*pnp*) with pSFR01 (with a ~23kDa prominent band), lane 4 shows protein profile of BL21 (Δ*pnp*) with pET22b (vector control), lane 5 shows protein profile of BL21 (Δ*pnp*) with pSFR02 and lane 6 shows protein profile of BL21 (Δ*pnp*) with pMC109 (with a ~100kDa prominent band) (positive control).

Figure 12 shows western blot pictures showing expression of PNPase, thioredoxin, poly-histidine or β-lactamase from plasmid constructs in *E. coli* BL21 (Δ*pnp*).

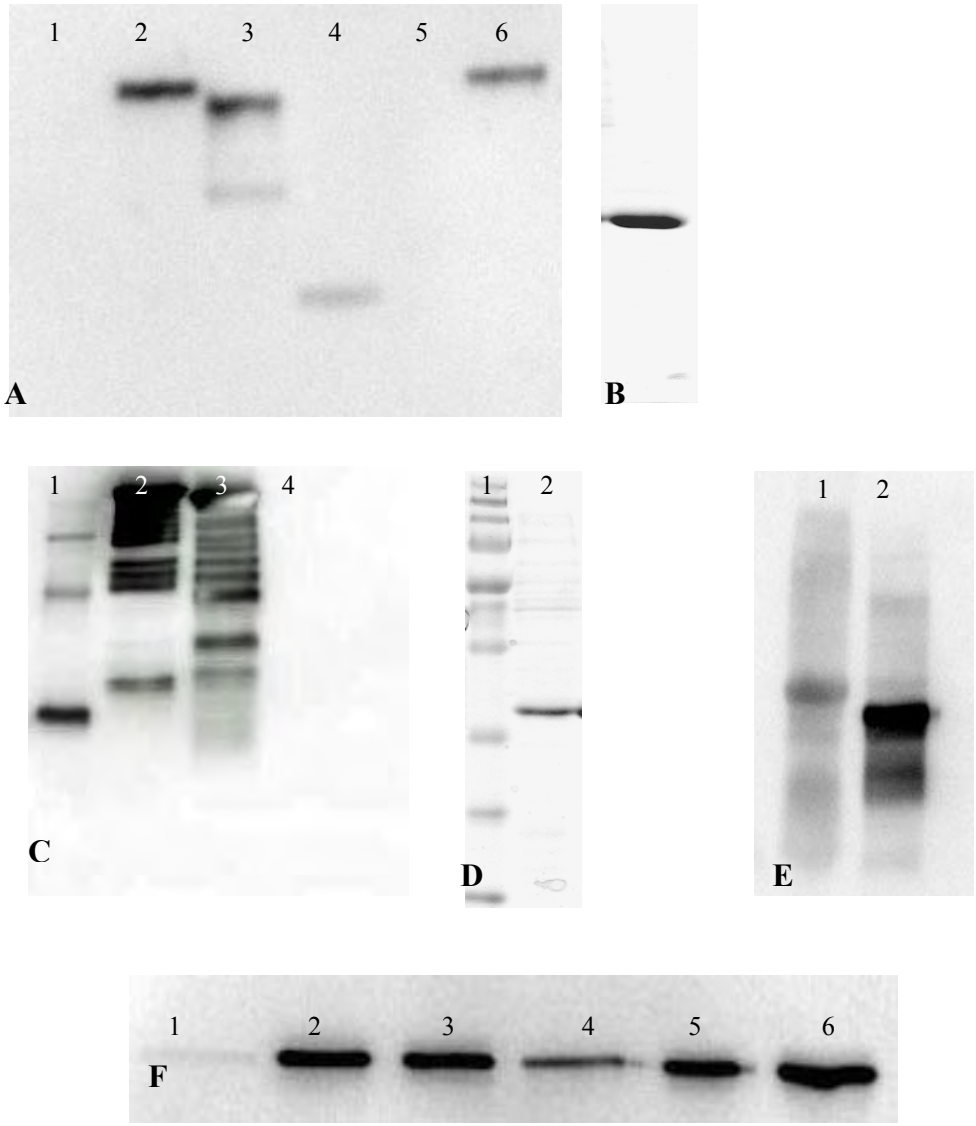

Fig. 12 Western blot analysis of the plasmid constructs. A Expression of PNPase detected with polyclonal rabbit anti-PNPase primary antibody. Lane a shows BL21 (Δ*pnp*) with pET32a (negative control), lane 2 shows BL21 (Δ*pnp*) with pMC109 (positive control), lane 3 shows BL21 (Δ*pnp*) with pMC110, lane 4 shows BL21 (Δ*pnp*) with pSFR01, lane 5 shows BL21 (Δ*pnp*) with pSFR02 and lane 6 shows BL21 (Δ*pnp*) with pMC109. **B.** Expression of s1 domain of PNPase with pSFR03, detected with polyclonal rabbit anti-PNPase primary antibody. **C** Expression of poly-histidine tagged PNPase detected with anti-His-tag HRP conjugate kit (Qiagen)**.** Lane 1 shows the 6xHis Protein ladder (Qiagen) (positive control), lane 2 shows BL21 (Δ*pnp*) with pMC109, lane 3 shows BL21 (Δ*pnp*) with pMC110, lane 4 shows BL21 (Δ*pnp*) with pSFR02. **D** Expression of poly-histidine tagged PNPase detected with anti-His-tag HRP conjugate kit (Qiagen)**.** Lane 1 shows the 'Poly-His Protein ladder' (Qiagen) (positive control), lane 2 shows BL21 (Δ*pnp*) with pSFR03. **E** Expression of a thioredoxin detected with primary polyclonal rabbit anti-thioredoxin antibody (Sigma). Lane 1 shows *E. coli* BL21 (Δ*pnp*) carrying pSFR03 and with pSFR01 in lane 2. **F.** Expression of β-lactamase with rabbit anti-β-lactamase primary antibody (Sigma). Lane 1 shows *E. coli* BL21 (Δ*pnp*) with pET32a, Lane 2 shows *E. coli* BL21 (Δ*pnp*) with pSFR01, Lane 3 shows *E. coli* BL21 (Δ*pnp*) with pSFR02, Lane 4 shows *E. coli* BL21 (Δ*pnp*) with pSFR03, Lane 5 shows *E. coli* BL21 (Δ*pnp*) with pMC109 and Lane 6 shows *E. coli* BL21 (Δ*pnp*) with pMC110.

β-galactosidase assay

To test effects of the *pnp* plasmid constructs on the *S.* Typhimurium virulence related genetic traits, the *S.* Typhimurium pSLT virulence plasmid (pEX102) that also encodes the major transcriptional activator for *spvA* expression, SpvR was transformed into *E.coli* TG1 strain. Under normal growth condition, in *E. coli* with a *pnp* proficient background, the S1 domain protein (pSFR01) showed higher transcriptional up-regulation of *spvA* compared to whole PNPase (pMC109) and the S1 deficient PNPase (pMC110). The plasmid pET32a served as a vector control (Figure 13).

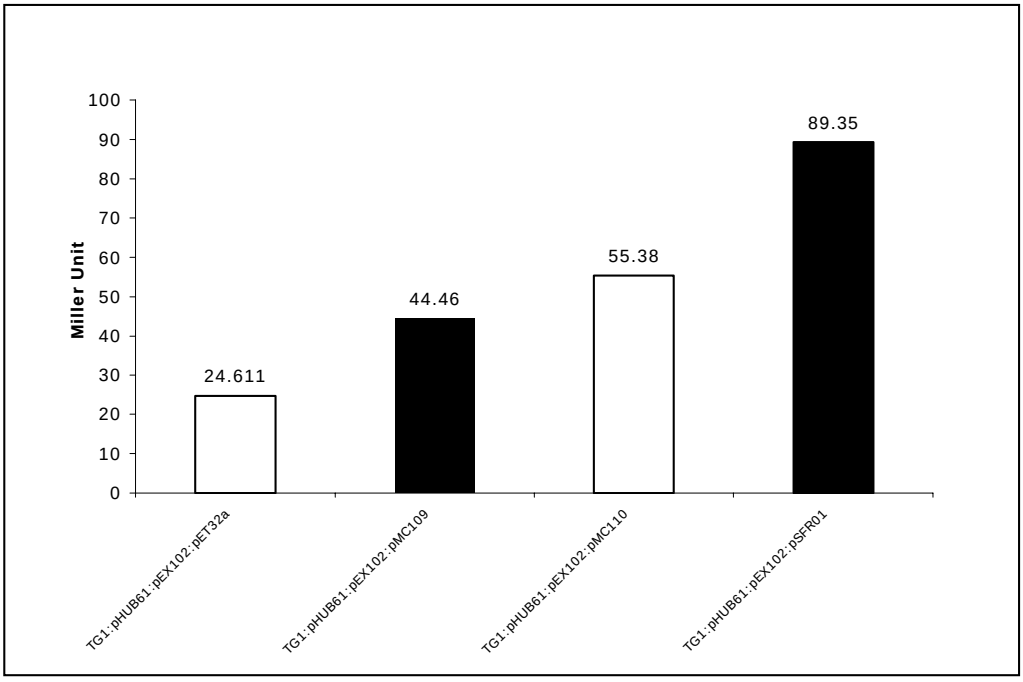

Fig. 13 β-galactosidase activity assays in *E. coli* (TG1) strains with different background. Bar graph shows effect of *pnp* and its different truncated forms on transcription of *spvA* through *spvA-lacZ* protein encoded by reporter plasmid pHUB61.

To check the effect on *spvA* promoter activity by different plasmid constructs in *S.* Typhimurium, cells were cultured in a low-pH minimal medium (MM5.8) that imitates the intravacuolar environment where the replication of *Salmonella* occurs[18]. In *S.* Typhimurium MC1 strain, no significantly different effect of the plasmid constructs was visible (Figure 14). The S1 domain (pSFR01) showed more or less similar effect as the whole PNPase (pMC109). Lack of the S1 domain (pMC110) showed a slight decrease on *spv* expression. But in a *pnp* deficient background (MC84), the S1 domain (pSFR01) upregulated *spv* expression more than whole PNPase (pMC109) while absence of S1 domain (pMC110) resulted in a sharp decrease in *spv* expression (Figure 14). The plasmid pET32a served as vector control in both cases. So, in *S.* Typhimurium, the S1 domain decreased the expression of *spv* genes in a *pnp* mutant under infection-simulating conditions.

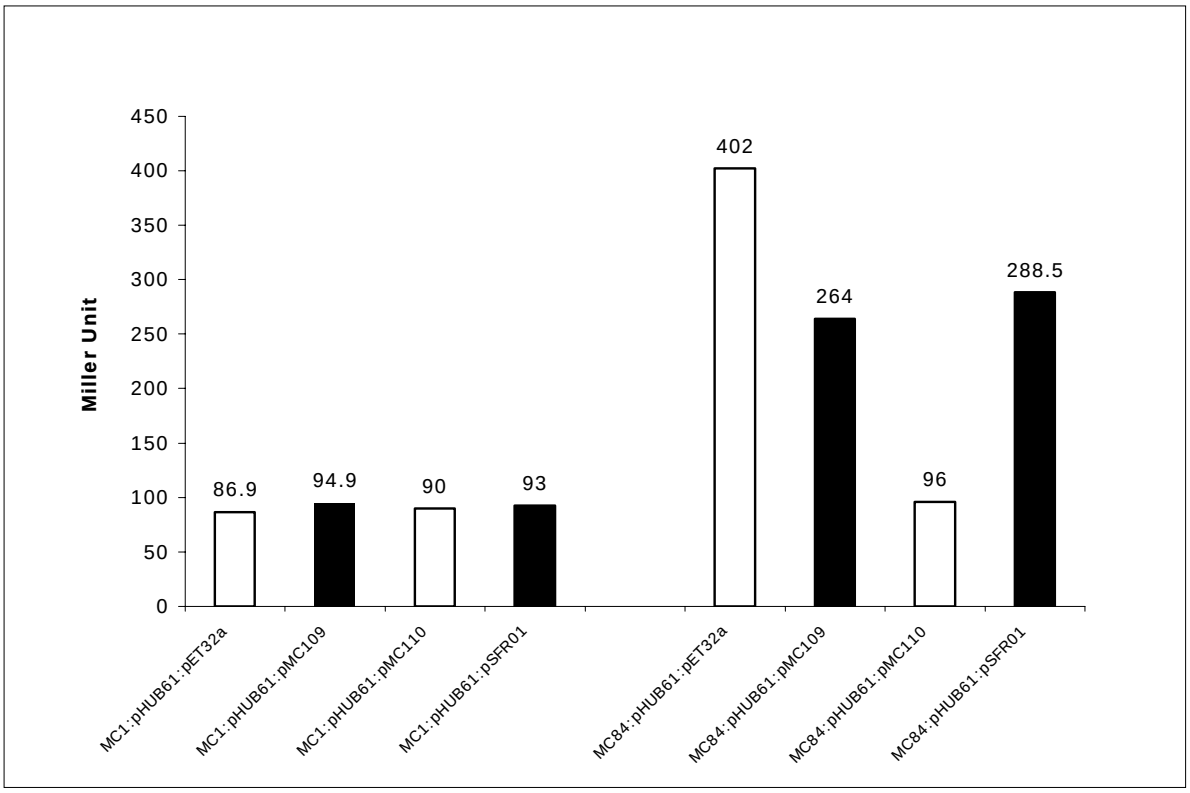

Fig. 14 β-galactosidase activity assays in *S.* Typhimurium (MC1, MC71 and MC84) strains with different background. Bar graph shows effect of *pnp* and its different truncated forms on transcription of *spvA* through β-galactosidase activity assays of *spvA-lacZ* protein encoded by reporter plasmid pHUB61 in *S.* Typhimurium *pnp*-proficient (MC1) and *pnp*-deficient (MC84) background.

DISCUSSION

The findings of PNPase S1 domain's sole effect on *Yersinia* T3SS activity [39] hence its virulence that resembles very much with *Salmonella*, the question that remained to be resolved is whether the S1 domain of the *Salmonella* PNPase alone is responsible for the regulatory constrain on its virulence or not. My study was mainly aimed to initiate the primary research to dissect the influence of *pnp* on *Salmonella* virulence gene expression. To do so, I constructed recombinant s1(*pnp*) plasmid(s), checked their expression and measured their effect on *Salmonella* plasmid virulence gene (*spvA*).

Constructing the recombinant plasmids

A first attempt to construct a C- terminally His_6- tagged S1 domain (codon 621-711 of *pnp*) was cloned in pET22b (pSFR02). However, no significant expression was found for the recombinant S1-His_6 protein. Fusion of thioredoxin (Trx-a 109 amino acid, 11.7kDa protein) at N or C terminal has been reported to improve the stability of recombinant proteins [10]. On this basis, a recombinant plasmid pSFR01 was constructed in vector pET32a(+) having a Trx fused S1 domain (codon 621-712 of *pnp*). The recombinant Trx-S1 protein was successfully expressed by western blot. To improve the purification of the recombinant S1 protein, a third plasmid was constructed from pSFR01 and pSFR02 having both His_6- and Trx- tagged (pSFR03).

Effect of PNPase-s1 domain on *spv(A)*

Effect of the S1 domain of PNPase on the expression of *spv* promoter was analyzed using an *spvA-lacZ* plasmid construct (pHUB61)[14] both in *E. coli* (TG1) and *S.* Typhimurium (MC1, MC84) under normal and infection-simulating conditions. To mimic the *S.* Typhimurium virulence related genetic traits, the *S.* Typhimurium virulence plasmid (pEX102) that also encodes the major transcriptional activator for *spvA* expression, *spvR* was transformed in *E.coli* TG1 strain.

PNPase is auto regulated [37] and mutations in its RNA-binding domains (KH and S1), can hamper this feature [16]. It has been shown also that expression of the virulence-associated *spv* gene cluster became highly increased when a PNPase-deficient mutant was grown under infection-simulating conditions [47]. The *E. coli* TG1 *pnp* shows 100% amino acid identity and 99% nucleotide identity to the *S.* Typhimurium *pnp* gene. In *E. coli* TG1 which carries a *pnp* wild-type, an over-expression of *pnp* (by genome and pMC109) lowered the expression PNPase by autoregulation under normal growth condition (LB, 37ºC, overnight). So, with the pMC109, the *spvA* expression was reduced (Fig. 13). Without the RNA-binding S1 domain, the PNPase lost its negative effect on *spv*, hence the *spvA* expression was increased with pMC110 (Fig. 13). In the presence of wild type *pnp* in *E. coli* TG1, the S1 domain encoded by pSFR01 up-regulated the *spvA* expression (Fig. 13). The *E. coli* TG1 with pHUB61, pEX102 and pET32a served as vector control (Fig. 13).

In *S.* Typhimurium the effects of the plasmid constructs were assessed both in *pnp*-proficient (MC1) and *pnp*-deficient (MC84) backgrounds. To analyze the impact of PNPase on *spv* using pHUB61, *Salmonella* was grown in a low-pH minimal medium (MM5.8). This medium

was designed to imitate the intravacuolar environment where the replication of *Salmonella* occurs [18]. In *S.* Typhimurium MC1, no significantly different effect were found between the effects of full-length PNPase (pMC109) and its truncated forms (pMC110 and pSFR01) on *spvA* expression (Fig. 14). The genomic PNPase might influence the effects of the plasmid products. Difference in the effects on *spvA* expressions by the plasmid constructs in *pnp* proficient *S.* Typhimurium MC1 and *E. coli* TG1 indicated the requirement of additional control element which might be absent in *E. coli*. So, the results showed that that the S1 domain is not neutral in terms of *spv* gene expression.

The effects of the plasmid constructs found different in *pnp* deficient *S.* Typhimurium MC84. In absence of chromosomal *pnp*, the full-length PNPase (pMC109) decrease the *spvA* expression as reported by Ygberg *et al*. [47] (Fig. 14). Interestingly, in absence of the RNA binding S1 domain (pMC110), this down-regulation was found even stronger (Fig. 14). One postulation behind this was lacking of the S1 domain might hamper the auto regulation of PNPase that might result with stable PNPase hence lowering the *spvA* expression. Only the S1 domain (pSFR01) also found to be able to down-regulate the *spvA* expression (Fig. 14). And this down regulation was almost as efficient as the whole PNPase (pMC109). Besides acting as a RNA binding domain, S1 domain of *pnp* has also catalytic activity. The *S.* Typhimurium MC84 with pHUB61 and pET32a plasmids served as a vector control for this experiment.

Combined, my data suggest that, the S1 domain of *S. Typhimurium* PNPase has profound effect on the *Salmonella* plasmid virulence (*spv*) genes in the intravacuolar environment. In *pnp* mutant, only the S1 domain of *pnp* is able to control the *spvA* expression level negatively almost as efficiently as the whole PNPase.

MATERIALS AND METHODS

The bacterial strains and plasmids and growth condition

Table 1 lists plasmids and genotypes of all bacterial strains used in this study. All of the strains and plasmids were used from Mikael Rhen's laboratory stock.

Table 1: Strains and plasmids

Strain or plasmid		*Relevant genotype or description*	*Source or Reference*
S. Typhimurium	MC1	Wild type SR-11 strain[A]	18
	MC84	MC1Δ*pnp*[B]	Mark Clements
E. coli	BL21Δ*pnp* (DE3)	B; (DE3), *pnp::Tn5 ompT gal dcm lon hsdS*$_B$ [C]	Novagen®
	TG1	K-12; *lac-, pro, supE, thi hsdD5* (F' *traD*36 *proA*$^+$*B*$^+$*lacI*^{q}*lacZ*M15	Gibson. T.J. 1984. PhD thesis. Cambridge University,UK.
Plasmid			
	pMC109	pET21c:: *pnp* (codon 1-711) cloned between *Bam*HI and *Xho*I, 3´ His-tag, Ampicillin^r	18
	pMC110	pET21c::5'*pnp* (codon 1-614) cloned between *Bam*HI and *Xho*I, 3´ His-tag, Ampicillin^r	18
	pET32a(+)	pET-based cloning and expression vector, T7 promoter, Trx-tag, His-Tag, Ampicillin^r	Novagen® (Appendix I)
	pSFR01	pET32a::*pnp* S1 region (codon 621-712), cloned between *Msc*I and *Xho*I, 5´ TrxA-tag, Ampicillin^r	This study
	pET22b(+)	pET-based cloning and expression vector,T7 promoter, His-Tag, Ampicillin^r	Novagen® (Appendix I)
	pSFR02	pET22b::*pnp* 1 region (codon 621-711), cloned between *Xba*I and *Xho*I, 3´ His_6-tag, Ampicillin^r	This study
	pSFR03	pET32a::*pnp* s1 region (codon 621-711), cloned between *Msc*I and *Xho*I, 5´ TrxA-tag, 3´ His_6-tag, Ampicillin^r	This study
	pHUB61	*spvA-lacZ* in cloning vector pACYC184, Chloramphenicol^r	47
	pEX102	The pSLT virulence plasmid of strain *S.* Typhimurium SL1265, Tetracycline^r	42

[A] A highly virulent strain, suitable for virulence assay.
[B] Nonsense codon at the beginning of the *pnp* reading frame.
[C] DE3 is a λ prophage expressing the T7 RNA polymerase under the control of a *lac* promoter. The *pnp* mutation is caused by an insertion of Tn5. The *hsdS*$_B$ defiency renders the strain deficient in both restriction and methylation due to the Hsd_B system

All *S.* Typhimurium and *E. coli* strains were cultured in Luria-Bertani broth (LB) and Luria-Bertani agar (LA) (Bacto Tryptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L, with or without Bacto agar 15 g/L) at 37ºC with or without appropriate antibiotics (ampicillin, 100 μg/ml; chloramphenicol, 10 μg/ml; tetracycline, 10μg/ml) as required. To analyze the impact of PNPase on *spv* (through β-galactosidase assay), *Salmonella* were cultured in a low-pH minimal medium (MM5.8), containing 100 mM Bis/Tris buffer (pH 5.6 with HCl) (Sigma),

0.1% (w/v) casamino acids (Sigma), 0.16% (w/v) glycerol, and 10 µM $MgCl_2$[18]. The medium imitates the intravacuolar environment where the replication of *Salmonella* occurs [18]. For every new experiment, I did fresh subcultures from original stock frozen at -70ºC.

Oligonucleotides

The *S.* Typhimurium LT2 genome sequence, available at the NCBI, NIH website [32], was searched for the open reading frame (ORF) of the *pnp* gene (sequence region 3449247-3451382, 2136bp). For the primer designing, I additionally used the online 'Oligo Calc: Oligonucleotide Properties Calculator' (Oligo Calculator version 3.21) [33] provided by Northwestern University, Chicago, USA. To amplify and clone the s1 domain (codon 621-712) of *pnp* (codon 1-712), I designed two sets of primers, Set 1, forward (PNP1) and reverse (PNP2) and Set 2, forward (PNP3) and reverse (PNP4). The primers also contained suitable restriction sites to clone into plasmid vectors pET32a (Novagen) and pET22b (Novagen) respectively. The design of the primers PNP1 and PNP4 also enabled them to have thioredoxin (Trx) and histidine (His) tagged in-frame inside the relevant vectors at the N and C terminal respectively. Table 2 shows the primer constructs used in this study.

Table 2: Primers used in the study

Primer		***Sequence***	***Restriction enzyme***
Set 1	PNP1	CGCTGGCCAAGTCGGCCGTATCTACAAT	*Msc*I
	PNP2	CCG CTCGAG TTACTCGGCCTGTTCGCT	*Xho*1
Set 2	PNP3	CTAGTCTAGATTTAAGAAGGAGATATACATGGTCGG CCGTATCTACAAT	*Xba*1
	PNP4	CCGCTCGAGCGGCTCGGCCTGTTCGCTCGC	*Xho*1

Restriction sites are underlined

Polymerase Chain Reaction

Standard polymerase chain reactions (PCR) were performed using a conventional thermocycler (Eppendorf Mastercycler®) according to the manufacturer's protocol using *Taq* DNA polymerase by Invitrogen™ (Cat # 18038-034). After an initial incubation at 95º C for 2 minutes the amplification was carried out in 30 cycles consisting of denaturation at 95º C for 1 minute, annealing at 60º C for 30 seconds and extension at 72º C for 2 minutes, followed by an additional incubation at 72º C for 2 minutes and then maintaining the reaction at 4º C. I separately used the primers Set 1 (PNP1 and PNP2) and Set 2 (PNP3 and PNP4) to amplify s1 region of *pnp* from the genomic DNA template extracted from MC1 strain. The amplified products (295 and 316 bp respectively) were analyzed by agarose gel electrophoresis.

Agarose gel electrophoresis

To analyze DNA (plasmid or PCR products), samples were run in agarose gels with different concentrations (0.6% -1.5%) stained with 0.5 µg/ml ethidium bromide in 1X TBE buffer (Tris-

borate-EDTA: 90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8.0) at 110 V and visualized by a Bio-Rad gel-documentation machine.

Restriction endonuclease cleavage reaction and ligation

Cleavage reactions were performed overnight in buffers shown in table 3 at 37ºC, according to the guidelines from New England Biolabs®.

Table 3: Reaction condition for restriction endonuclease cleavage

Enzyme	***Buffer***
*Msc*I	Neb 4
*Xho*I	Neb 4, Neb 2
*Msc*I +*Xho*I	Neb 4
*Xba*I	Neb 2
*Xba*I + *Xho*I	Neb 2
*Pst*I	Neb2

Cleaved PCR amplicons and vectors were ligated with T4 DNA Ligase and 1X T4 DNA Ligation reaction buffer supplied by New England Biolabs® at 16ºC, overnight as described by the supplier. Cleaved and ligated products were analyzed by agarose gel electrophoresis.

DNA Extraction

To extract DNA, different types of commercial kits were used depending on the types of preparation for e.g. PCR products, genomic DNA etc. PCR products were purified using the 'QIAquick PCR purification kit (28104)' by Qiagen. For genomic DNA extraction, 'Wizard genomic DNA purification Kit' (Promega) was used. To extract DNA from agarose gel, the bands of expected size in the agarose gel were cut and extracted using 'QIAquick gel extraction kit' by Qiagen. For plasmid extraction, 'QIAprep spin miniprep kit' (Qiagen) was used.

Preparation of competent cells

Cells were first cultured on LA (with or without antibiotic) overnight at 37ºC. Subcultures were grown with agitation in 10 ml LB (with or without antibiotic) at 37ºC for 3 hours. Cells were then centrifuged at 4500g for 10 minutes. Pellets were carefully re-suspended in 2 ml ice-cold 0.1M $CaCl_2$ and incubated overnight at 4ºC.

Transformation of the plasmids

Plasmids were transformed into competent cells using chemical transformation method by Wesink *et al.* (1974) [45]. A 100 µl suspension of competent cells was mixed with 5-10µl plasmid and incubated on ice for 20 minutes. The suspension was heat shocked at 42ºC for 2 minutes, followed by addition of 1 ml LB and 2-3 hour incubation at 37ºC and subsequently plating on LA with appropriate antibiotics. The plates were incubated at 37ºC, overnight,

resulting single transformed colonies. Colonies were subcultured by re-streaking to get single transformed isolates.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide (acrylamide: bisacrylamide 29: 1 from Bio-Rad) gel electrophoresis (SDS-PAGE) was carried out to analyze the protein expression. For SDS PAGE, streak plate cultures on LA with required antibiotics were first incubated for overnight at 37ºC. An isolated single colony was then inoculated in 10 ml LB with required antibiotics and cultured overnight with shaking at 37ºC. From each culture, a 100 µl suspension (OD_{600}= 2) was re-inoculated into 10 ml LB with required antibiotic(s) and isopropyl β-D-1-thiogalactopyranoside, IPTG (1 mM) that triggers the expression of T7 RNA polymerase and incubated at 37ºC with shaking for 3 hours. At OD_{600}= 0.8, 30 µl was taken from each culture into a 1.5 ml microcentrifuge tube and mixed with 15 µl 2X sample buffer (0.09M Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 0.02% bromophenol blue and 0.1M dithiothreitol, [DTT]) and heated at 95°C for 15 minutes. The peptides were separated by an SDS PAGE gel as described by Laemmli *et al.*(1970)[21] with 12% separation gel (0.36 M Tris-HCl pH 8.8, 0.1% SDS, 39.9% of 30% acrylamide: bisacrylamide 29: 1, 0.5% of 10% ammonium peroxodisulphate [APS] and 0.005% of N,N,N',N'-Tetramethylethylenediamine [TEMED]) and a 4% stacking gel (0.115 M Tris-HCl pH 6.8, 0.1% SDS, 13.3% of 30% acrylamide: bisacrylamide 29: 1, 0.99% of 10% APS and 0.099% of TEMED). The gel was run in 1X SDS running buffer (25 mM Tris base, 192 mM glycine and 0.1% SDS, pH 8.3) using the Bio-Rad 'Mini Protean' electrophoresis system at 200 V. It was analyzed through Coomassie blue staining by submerging the gel with the staining solution (20% methanol, 10% acetic acid, 0.1% Coomassie Brilliant Blue G) for 1 hour with agitation and subsequent shaking with de-staining solution (20% methanol 70% water and 10% acetic acid) for 30 minutes.

Western blot

Western blotting procedures were carried out as described in QIAexpress® Detection Assay Handbook (Qiagen, October, 2002). For blotting, the proteins were transferred to a hydrophobic polyvinylidene difluoride (PVDF) (Hybond™ P; Amersham). Table 4 summarizes the antibodies used for this study. Detection of poly-histidine was performed according to the manufacturer's guideline using the Anti-His HRP conjugate kit (Qiagen).

Table 4: Antibodies

Detection	***Primary Antibodies***	***Secondary Antibodies***
PNPase (full length or truncated forms)	Rabbit (polyclonal) anti-PNPase antibodies (1:500)[A]	Stabilized goat anti-rabbit-IgG antibodies conjugated with horse radish peroxidase, HRP [Pierce][B] (1:500)
Thioredoxin	Rabbit anti-thioredoxin antibodies [Sigma] (1:5000)	Do
β-lactamase	Rabbit anti β-lactamase antibodies [Sigma] (1:5000)	Do

[A] Dilution applied
[B] Source

All the signals were generated through chemiluminescence process using a Supersignal West Dura Extended Duration Kit (Pierce) and recorded using the LAS-1000 System (FUJIFILM) and IMAGEQUANT software (version 5.2).

Protein purification

For purification of Trx-S1-His_6 recombinant protein encoded by pSFR03, a loopful of cells were re-suspended from an overnight plate culture into 10 ml LB with ampicillin containing 1 mM IPTG to an OD_{600} of about 0.5. The culture was grown for one hour on a shaker at 37°C, 180 rpm, centrifuged down at 4000 g and re-suspended in 10 mM Tris-HCl, pH 8.0 and frozen over night at -70°C. The next day, the cells were thawed, sonicated for three minutes with 30 seconds interval on ice in a Soniprep 150 sonicator (Sanyo) at 15 microns amplitude, and the insoluble debris were removed by centrifugation at 8000 g in a Sorvall SS-34 rotor for 30 minutes at 4°C. The lysate was then processed according to the instructions that followed a Qiagen Ni-NTA Spin Kit. Purified protein was then mixed 1:1 with 1X SDS-PAGE sample buffer and run on a 12% SDS-PAGE gel.

Determination of β-galactosidase activities

β-galactosidase activities expressed by *lacZ* transcriptional construct were measured using ortho-nitrophenyl-β-D-galactopyranoside (ONPG) (Sigma) as a substrate[49]. Overnight cultures in LA with antibiotics were subcultured in 10 ml of LB (plus antibiotics) and grown overnight at 37°C with agitation. Reaction was carried out in 2 ml sterile micro-centrifuge tube (Eppendorf) having 700 µl Z-buffer (0.06 M $Na_2HPO_4.7H_2O$, 0.04 M $NaH_2PO_4.H_2O$, 0.01 M KCl, 0.001 M $MgSO_4$, 0.05 M β-mercaptoethanol, pH 7.0) and 100 µl bacterial suspension. To permeabilize cells, 40 µl of 0.1% SDS and 40 µl of chloroform were added. Then 0.2 ml ONPG (4 mg/ml) was added to each tube and the sample vortexed to initiate the reaction. The tubes were then placed in a 30°C thermoblock and the time was noted. The reaction was stopped by adding 0.5 ml of 1 M Na_2CO_3 when a yellow color developed. The time was noted again when each reaction was stopped. The tubes were vortexed and left on the benchtop for 15 minutes. The absorbance was measured at OD_{420} and OD_{550} for each reaction tube and OD_{600} of the corresponding cell suspension. The β -galactosidase activity of each sample was calculated using the formula: Muller Units = $1000\ \{[OD_{420} - (1.75 \times OD_{550})]\ /t \times V \times OD_{660}\}$ with t = reaction time in min; V = volume of cell suspension in assay in ml; OD_{420} and OD_{550} of reaction solution; OD_{660} of original cell suspension. All measurements were done in triplicates.

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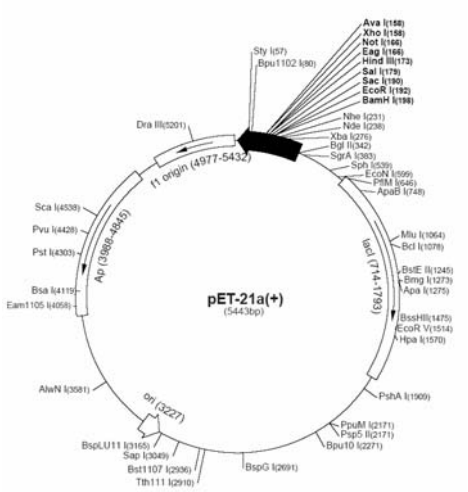
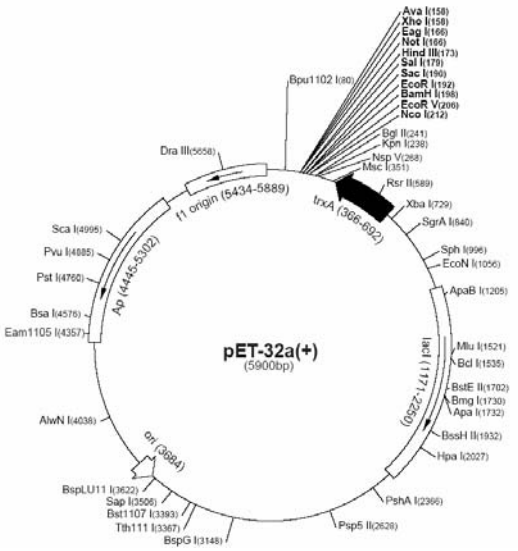
APPENDIX

pET-21a(+) sequence landmarks	
T7 promoter	311-327
T7 transcription start	310
T7•Tag coding sequence	207-239
Multiple cloning sites	
(*Bam*H I - *Xho* I)	158-203
His•Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	714-1793
pBR322 origin	3227
bla coding sequence	3988-4845
f1 origin	4977-5432



pET-32a(+) sequence landmarks	
T7 promoter	764-780
T7 transcription start	763
Trx•Tag coding sequence	366-692
His•Tag coding sequence	327-344
S•Tag coding sequence	249-293
Multiple cloning sites	
(*Nco* I - *Xho* I)	158-217
His•Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	1171-2250
pBR322 origin	3684
bla coding sequence	4445-5302
f1 origin	5434-5889



pET-22b(+) sequence landmarks	
T7 promoter	361-377
T7 transcription start	360
pelB coding sequence	224-289
Multiple cloning sites	
(*Nco* I - *Xho* I)	158-225
His•Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	764-1843
pBR322 origin	3277
bla coding sequence	4038-4895
f1 origin	5027-5482

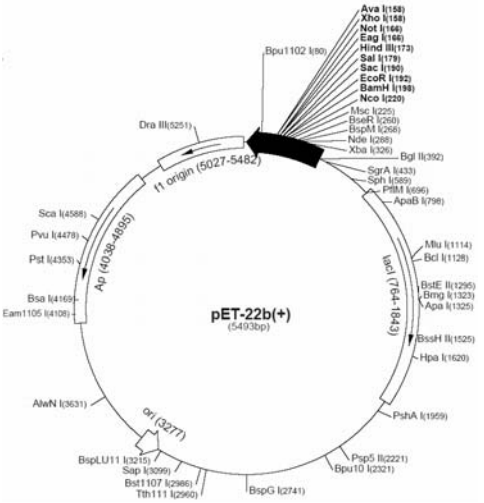


Fig.15 Maps of the plasmid vector pET21a(+), pET32a(+) and pET22b(+) showing restriction sites and sequence landmarks. [Courtesy Merck-Novagen [29], published for non commercial use]