Formation of *slt* II:: Tn*pho* A gene fusion

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Abstracts

The structural genes for Shiga-like toxin (*slt* A and *slt* B) in *Escherichia coli* appear to be transcribed as an operon from a promoter upstream of *slt* A. The *slt* Gene was the target gene wants to study or manipulated using a transposable element that carries the *pho*A gene, which encodes alkaline phosphatase enzyme, this enzyme can be used as a reporter for *slt*-II gene expression and protein localization. In this study we used gene fusion techniques, to form a *slt*-II ::*Tnpho*A fusion, attempting to make a gene fusion between the promoter and proximal portion of *s1t* A gene with the gene for bacterial alkaline 2 phosphatase to assess the regulation of Shiga-like toxin expression, and gene expression, protein localization, and functional characterization of *slt*-II genes. A gene fusion between the *slt*-ll operon and the transposon vector *Tnpho*A, such that expression of the *pho*A gene was brought under the control of the slt-II promoter, was then developed. The presence and site of insertion of *Tnpho*A within the operon was confirmed by restriction analysis. Use of the resulting single copy fusion derivative and a plasmid encoded a *slt*-II::*Tnpho*A fusion demonstrated significant differences in the synthesis, secretion and localisation abilities of *slt*-II during growth. These characteristics could have important implications on the relative abilities of the toxins to cause disease *in vivo.* This specific type of fusion involves combining the *slt* gene with the *Tnpho*A transposon, which includes the *pho*A gene. We used the construction derivative of transposon TnS that permits the generation of hybrid proteins composed of alkaline phosphatase lacking its signal peptide fused to amino-terminal sequences of other proteins. Such a hybrid gives alkaline phosphatase activity if the protein fused to alkaline phosphatase contributes sequences that promote export and thus compensate for the missing alkaline phosphatase signal peptide. We explained of how such a gene fusion can be formed. Plasmid NTP707 contains a 4.7 Kb *Eco*RI fragment encoding the entire *slt*-II operon, inserted into the chloramphenicol resistance (Cmr) gene of plasmid pACYC184. The recombinant plasmid NTP707 (Willshaw *et al,,* 1987) was electroporated into *E. coli* CC118 and transformants selected on LB+Tc plates. *Tnpho*A was introduced by transduction of a log phase culture of CC118 (pNTP707) with lambda *Tnpho*A (Gutierrez *et al.,* 1987). Growth of transformants on LB+Tc+Kn (300)+XP plates selected for transposition of *Tnpho*A onto the high copy number plasmid vector. CC118 was retransformed with plasmid preparations of the transformants and blue colonies on LB+Tc+Kn (50)+XP plates isolated. These contained in-frame fusions of *Tnpho*A to secreted gene products on plasmid NTP707. Plasmid DNA was prepared from transformants displaying Tc resistance (Tcr), restricted with *Sma*I-*Pst*I and electrophoresed on an agarose gel. Restriction with *Sma*l-*Pst*l produced the expected 8 and 0.85 Kb fragments (figure 6), confirming the presence of pNTP707 within *E. coli* CC118. Both the plasmid containing the *slt*-II gene and the *Tnpho*A transposon vector are digested with restriction enzymes to create compatible ends for ligation. To more finely define the site of insertion of *Tnpho*A within this fusion plasmid, this plasmid was restriction mapped using single and multiple restriction endonucleases. The enzymes used were *Eco*RI, *Eco*RV, *Pst*l, *Smal*. It became evident almost immediately from the pattern of *Eco*RI digested plasmid DNA (figure 6), that the location of the fusion joint was not randomly distributed over the whole slt-II operon. In the formation of *a slt::Tnpho*A gene fusion (figure 13), both the A and B subunits are synthesised with amino terminal signal sequences. *Tnpho*A can therefore fuse into either subunit gene and be exported into the periplasm to give enzyme activity. As both genes are transcribed from a single promoter upstream of the A subunit gene in *slt*-II both possible gene fusions allow monitoring of *slt-II* gene expression to be undertaken.

Introduction

Production of *slt*-I and *slt*-II genes has been shown to result from infection by lysogenic bacteriophage carrying the structural genes for toxin production (Scotland *et al.,* 1983). Gene fusion was found to be a potent process for evolutionary novelties in bacteria. For example, in 18 bacteria species, including *Escherichia coli*, it was found that 0.2–1% of the genes in each genome were fusion genes (Snel *et al.,* 2000). However, it has been a challenge to understand the role of gene fusion in multicellular eukaryotic organisms. Early medical genetic studies revealed that some cancer-genes mutations have arisen from the fusion of 2 adjacent genes in the genomes of cancer patients (Thomson *et al*., 2000), implicating the role of gene fusion as a mechanism for new gene evolution, even though such fused genes may have deleterious consequences upon their birth. Analyses of the origination of fusion genes in humans and other hominoids have shown that they can form at both the DNA (i.e., alternative splicing site skip or mutation, transposon element related movement or recombination) and RNA levels (i.e., retroposition with subsequent flanking sequence recruitment (Zhang Y *et al*., 2009) thereby revealing the diverse molecular processes that can lead to gene fusions. However, recent sequencing efforts in several model organisms have predicted that gene fusion is likely an active molecular process in eukaryotes. For example, in human it has been shown that at least 4–5% of tandemly duplicated genes are transcribed into single putative fusion transcripts (Goodstadt L *et al*., 2006). Individual subunits of Shiga Like Toxin -I and Shiga Like Toxin -II can assemble to form fully cytotoxic hybrid molecules in vitro, as recently demonstrated by Ito et al., (1988) with purified A and B subunits of the toxins.

Protein fusions have played a central role in molecular genetic studies of the mechanism of protein export in bacteria (Beckwith & Silhavy, 1983). Conventionally this approach involves the fusion of a selectable "reporter" gene, which possesses an easily assayed activity, to the promoter and controlling regions of the particular gene under study. The "reporter" gene may be used in either transcriptional (operon) fusions, where it retains its own translational start but is dependent on the attached DNA for transcription, or in translational protein fusions, where both transcription and translation are dependent on signals in the attached upstream DNA. Subsequently, Hoffman & Wright (1985) constructed a set of plasmids which encode the gene *(pho*A*)* for the *E. coli* periplasmic protein alkaline phosphatase (*Pho*A), missing its own signal sequence. Enough of the mature protein is retained such that highly active *Pho*A can be generated *in vitro* by attachment to *pho*A of appropriate restriction fragments containing the amino termini of other proteins. PhoA only displays activity when exported from the cytoplasm as it must dimerise to be active to allow the formation of the necessary disulphide bonds. Manoil & Beckwith (1985) extended this approach with the formation of *Tnpho*A, a transposon vector that allows generation of gene fusions between the amino terminal portion of a target gene and the coding sequence of bacterial PhoA *in vivo.* A derivative of Tn5, *Tnpho*A is composed of *pho*A lacking its promoter, its translation initiation site, and the DNA corresponding to the signal sequence and first five amino acids of the protein (fig. 1. a, b).

Fig 1. a. and b. The transposon vector *TnphoA* (Manoil & Beckwith, 1985) Detailed structure of *TnphoA*

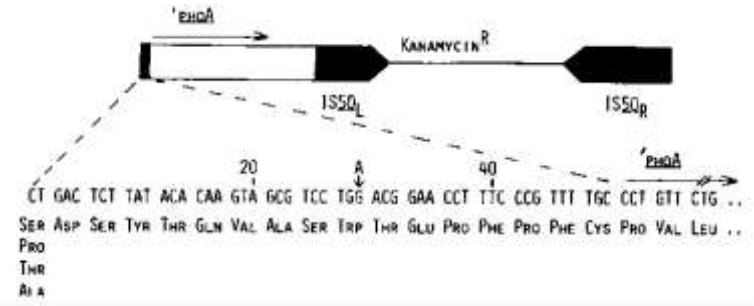

Fig 1 a. *There are 50 base pairs (bp) at the beginning of the phoA coding region. Of this sequence, 48 bp are derived from IS50L of transposon 'n5 and 2 bp originate from the Pstl linker of plasmid pCH39 (Hoffman & Wright, 1985). The amino acid residues that are shown are present at the ftision joint of every hybrid protein generated. The DNA sequence differs from that of the IS50L region of Tn5 by an A-to-G change at position 29. All of the phoA is encoded except the signal sequence and 5 amino acid residues of the mature protein (Inouye et al., 1982).*

b. Restriction enzyme map of *TnphoA*

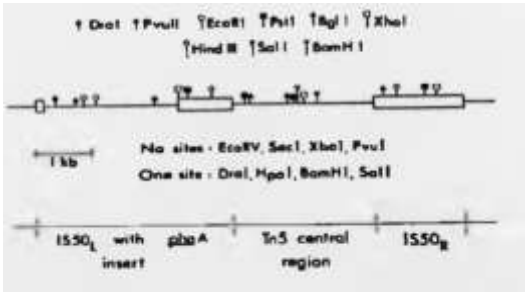

Fig. 1. b*. This figure presents the restriction enzyme map of the TnphoA transposon. The map details the locations of restriction enzyme cleavage sites within the TnphoA sequence, providing insight into the fragment sizes and potential sites for enzyme digestion. This information is crucial for understanding the structure and manipulation of TnphoA in genetic experiments*

There are 50 base pairs (bp) at the beginning of the phoA coding region. Of this sequence, 48 bp are derived from the IS50L of transposon Tn5 and 2 bp originate from the PstI linker of plasmid pCH39 (Hoffman & Wright, 1985). The amino acid residues are present at the fusion joint of every hybrid protein generated. The DNA sequence differs from that of the IS50L region of Tn5 by an A-to-G change at position 29. All of the *pho*A is encoded except the signal sequence and 5 amino acid residue of the mature protein (Inouye et al., 1982). The transposon is a derivative of Tn5 with a region encoding E. coli alkaline

phosphatase, minus the signal sequence and expression signals, inserted into the left IS50L element (Manoil & Beckwith, 1985). Active insertions into X interrupt the gene and result in production of a hybrid protein from the X-phoA fusion. (fig. 2)

Fig.2 The structure and restriction map of the transposon vector TnphoA (Manoil & Beckwith, 1985)

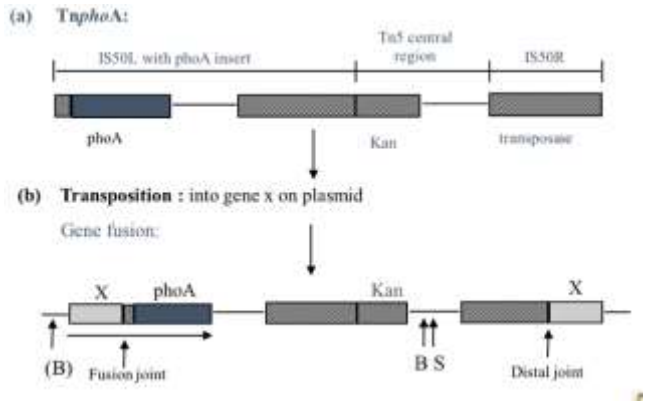

Fig. 2. *This figure presents the structural layout and restriction map of the TnphoA transposon vector. The map details the locations of various restriction sites within the transposon vector, providing a visual representation of its genetic elements and the positions of restriction enzyme cleavage sites. This diagram is essential for understanding the organization of TnphoA and facilitating its use in cloning and genetic analysis*

Hybrid proteins expressing PhoA activity only occur when *'Tnpho*A inserts in the proper orientation and reading frame to a signal that promotes the export of the protein from the cytoplasm. This signal can correspond to those found in periplasmic, outer membrane, cytoplasmic membrane or secreted proteins. The use of transposon *Tnpho*A combines the advantages of working with hybrid proteins able to be secreted (Hoffman & Wright, 1985), with the versatility of Tn5 transposition in generating hybrids (Bruijn & Lupski, 1984). PhoA is an easily assayable enzyme which can be detected even at low levels in bacterial colonies by the use of the chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate p-toluidine (XP). When PhoA hydrolyses XP in some appropriate agar medium E. coli colonies are blue. Use of the sensitive indicator media and enzyme assay for PhoA, also provides a simple way to monitor the expression of the hybrid gene, which in turn reflects the activity of the exogenous promoter. The use of *pho*A fusions has proven to be a powerful tool for the study of protein secretion, membrane protein topological structure, protein export signals and the identification of genes for cell envelope and

extracellularly secreted proteins (for a review, see Manoil *et al.*, 1990). Use of the sensitive indicator media and enzyme assay for PhoA, also provides a simple way to monitor the expression of the hybrid gene, which in turn reflects the activity of the exogenous promoter. Gene fusion technology has previously been exploited to assess the regulation of VT1 expression by iron (Calderwood & Mekalanos, 1987). This involved the creation of hybrid genes between the promoter and proximal portions of *slt-l*A and *TnphoA*. The *VT*-II*::Tnpho*A gene fusion plasmid, pSC105 was kindly provided by Dr S Calderwood, Harvard University, to enable study of *slt*-II gene expression. In order to quantitate *slt*-II gene expression, fusions between *TnphoA* and the cloned *sit*-II operon carried on plasmid NTP707 were made in this study. *Tnpho*A can therefore fuse into either subunit gene and be exported into the periplasm to give enzyme activity. As both genes are transcribed from a single promoter upstream of the A subunit gene in *slt*-II both possible gene fusions allow monitoring of *slt*-II gene expression to be undertaken. In this paper we present an extension of this approach that allows the ready isolation of fusions to alkaline phosphatase in vivo. We describe a transposon, TnphoA, that can insert into a gene to generate fusions of alkaline phosphatase to amino-terminal sequences of the protein product of that gene.

Aim: The purpose of this study was to develop a method to detect *slt*-II by monitoring the synthesis of the *slt*-II -TnphoA

Material and method

Table 1. Bacterial strains

Bacteria strains	Source	Reference
ATCC 35150	Warwick Hospital	S. Scotland S. Scotland T. Meyer T. Meyer
NCTC 8781	Warwick University	B. Rowe B. Rowe P. Reeves P. Reeves D. Cardy S. Colby
Carter et aL (1985)		
E. coli		
C600(933W)		
E. coli K12 60R746		
E. coli K12 60R363		
E. coli DH1		
E. coli CC118		
E.coli TGI		
S. dysenteriae		
Type 1		
E. coli CC118		

Table 2. Plasmid

Plasmids	Phenotype	Source	Reference
NTP705	Knr *slt*-IB' Knr j/i-IB'	B. Rowe	Willshaw *et al.* (1985)
NTP707	TC r *slt*-IB'.	B. Rowe	Willshaw *et al.* (1987)
pSC105(1987)	Ampr Kanr, *slt-lA::TnphaA*	S. Calderwood	Calderwood
pSC2.	Ampr. *slt-lA*		
Mekalanos			

pSLF22	Knr, *slt-II::TnphoA*	this study
pSLF34	Knr, *slt-II::TnphoA.*	*This study*
pACYC184		

Table 3. Bacteriophage

Phage	Source	Reference
Lambda *Vir.*	P. Reeves, Warwick University	
Lambda *TnphoA*	P. Reeves, Warwick University	Manoil & Beckwith (1985)

Isolation of plasmid DNA: The alkaline lysis technique of Birnboim & Doly (1979) was used as described by Maniatis *et al.* (1982) except that the volumes were reduced by 20% to allow the use of Oakridge tubes. Additionally, solution II (alkaline SDS solution) was not placed on ice prior to use (as the SDS precipitates) and a centrifugation step (39,200g, 30 min, 15°C) was included prior to the CsCl gradient centrifugation to remove cell debris. After removal of ethidium bromide, plasmid DNA was directly precipitated from CsCl a and resuspended in 500/11 of TE.

Restriction endonuclease digestion: Restriction endonucleases and restriction endonuclease buffers (xlO) were obtained from Amersham or BRL, and used according to the manufacturer's instructions.

Agarose gel electrophoresis: To prepare horizontal slab gels, 1% (w/v) agarose, unless otherwise stated, was melted in TBE electrophoresis buffer and cooled slightly before pouring. DNA samples, restricted as appropriate, were mixed with 1/6 volume of Loading Buffer Type IV and loaded into the gel slots. Electrophoresis was carried out with the gel completely submerged in buffer at 100-120 volts (V) or 60 V overnight. As DNA molecular weight (MW) markers, 10/d of lambda+ (bacteriophage DNA (50 ng ml'*), digested with the restriction endonuclease *Hindl,* was used. This generated fragment sizes: 23.17, 9.46, 6.75, 4.26, 2.20, 1.92 and 0.58 kilobases (Kb). DNA was stained within the agarose gel with ethidium bromide (0.5 *n*\ ml"*) as described by Maniatis *et al.* (1982), and visualised by transillumination with short-wave UV light and photographed using Polaroid Type 665 black and white film.

Bacterial transformation: Two methods were employed to introduce DNA into bacterial cells:- Electroporation and the induction with lambda *TnphoA*

Electroporation: Electroporation, one of the most recent advances for the introduction of DNA into cells, involves the application of a brief, high voltage pulse to a suspension of cells and DNA, resulting in a transient membrane permeability and the subsequent uptake of DNA. Cells for electroporation were propagated in 100 ml LB and harvested at an OD600nm of 0.6-0.8. Cells were pelleted in a Beckman 6x250 rotor (22,100g, 10 min, 4°C), the supernatant discarded and the cell pellet washed twice in 50 ml ice-cold SDW. The pellet was then washed in 25 ml ice-cold sterile 10% (v/v) glycerol, repelleted and resuspended in 200 *pA* of the latter. The cells were frozen on dry ice and stored at - 70°C. Electroporation was carried out using a Gene Pulser (Bio-Rad Labs USA), set to 2.5 KV, a capacitance of 25 *fi¥* and a resistance of 200 ohms. 1 */A* of CsCl density gradient purified plasmid DNA was mixed with 40 *pA* of electroporatable cells and left on ice for 1 min before being transferred to an ice-cold 0.2 cm electroporation cuvette. The cell/DNA mixture was spread evenly across the bottom between the chilled electrodes, the cuvette placed in the safety chamber and the pulse applied. Following the pulse, 1 ml of LB was added and the mixture transferred to an Eppendorf tube and incubated at 37°C for 1 hour to allow for expression of the antibiotic resistance genes. 100*fil* aliquots were then plated onto selective agar to screen for transformants.

Transduction with lambda *TnphoA*: Lambda *TnphoA* (table 3) was provided by Dr. G. Salmond, Dept, of Biological Sciences, Warwick University. A single transformant of *E. coli* CC118 (pNTP707) was grown overnight in LB/Tc and 100/d used to inoculate 10 ml fresh LB/Tc. This was grown to exponential phase at 37°C with shaking (250 rpm). The culture was pelleted in a MSE Mistral 1000 universal centrifuge (2,940g, 5 min, room temperature), the pelletresuspendedin1mlofLB/Mal/Mg Tcand 100*p* of lambda *TnphoA*was *add*ed. After static incubation at 37°C for 30 min to allow for phage adsorption and infection, 10 ml LB/T c was added and the culture incubated for 1 hour at 37°C (250 rpm) to allow for expression of Knr. The culture was pelleted as before, resuspended in 1 ml LB/Tc and 100*mug* aliquots spread on LB plates containing Tc, Kn (300 *mu*g ml'1) and XP (Sigma, 40 *fig* ml' 1 in DMSO) and incubated at 37°C for two days. Mini plasmid preparations of blue colonies were used to transform freshly competent *E.coli* CC118. Individual blue colonies on LB plates containing Tc, Kn (30 *mug* ml'1) and XP after 2 days at 37°C, contained in frame fusions of *TnphoA* to secreted gene products on plasmid NTP707.

Two methods were employed to introduce DNA into bacterial cells:- Electroporation and the induction of "Pseudocompetence" by pretreatment with Ca^+ .

The calcium chloride procedure: The technique used was essentially that described by Holland (1983). 0.5 ml of overnight culture was used to inoculate 50 ml of LB in a 100 ml flask. This culture was grown to an OD600nm of approximately 0.5 and the cells were then held on ice for 10 min. From this point, all operations were carried out on ice and using ice-cold solutions. The cells were pelleted by minimal centrifugation in a multex angled centrifuge, the supernatant carefully removed and the pellet resuspended in 0.5 volume of CaCl2 (100 mM). After a further 10 min on ice, the culture was repelleted, resuspended in 0.05 the original volume (2.5 ml) of 100 mM CaCl2 and was held on ice for a minimum of 1 hour (Dagert & Ehrlich, 1979). 200 *pi* of competent cells were aliquoted into a 1.5 ml Eppendorf tube and 1 *pi* DNA added to the cells, mixed gently and then left on ice for 30 min. This was then heat shocked at 42°C for 2 min and returned to ice for 15 min. Two volumes of LB were added, incubated for 1 hour at 37°C, to allow expression of antibiotic resistance genes after which 100 *p* \ aliquots were plated onto selective media.

Preparation of fragments from agarose gel: The excised fragment was placed in dialysis tubing (prepared as described by Maniatis *et al.,* 198) with a small volume of 0.5 x TBE (approximately 200 *fi\).* All air bubbles were removed and a current of 40 mA was applied across the dialysis tubing in a mini gel tank buffered with 1 x TBE for 20 min. The polarity was then reversed for 30 sec to remove any DNA from the side of the dialysis tubing and the DNA in solution removed to an Eppendorf tube. The solution was extracted twice with phenol, once with TE saturated chloroform and the DNA precipitated at -20°C for a minimum of 3 hours upon addition of 0.1 volumes 3 M Na Acetate and 2 volumes ethanol. DNA was recovered by centrifugation in a MSE Micro Centaur (ll,600g, 10 min, 4°C), washed with 70% (v/v) ethanol, repelleted and dried under vacuum for 5 min. The dried pellet was resuspended in 20 mgTE.

Results:

Restriction enzyme map of the VT2 operon (Jackson *et al*., 1987b)

Restriction sites are given within the 3250 bp *Sph*I to *Eco*RI fragment isolated from the hybrid plasmid pNN76. The location and orientation of the structural genes, *slt*-IIA and *slt*-IIB, are shown above the restriction map (fig. 3)

Fig. 3. Restriction Enzyme Map of the VT2 Operon (Jackson et al., 1987b)

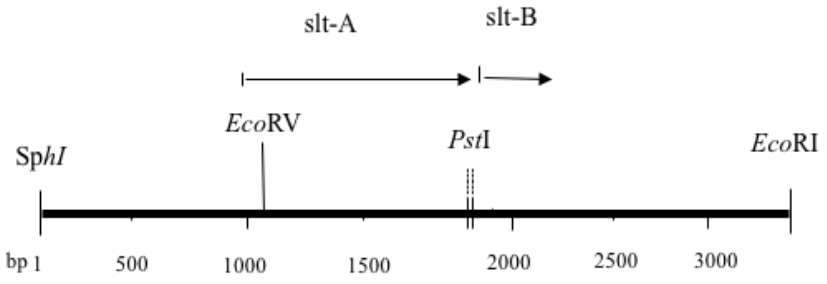

Fig. 3. *This figure displays the restriction enzyme map of the VT2 operon, derived from the 3250 bp SphI to EcoRI fragment isolated from the hybrid plasmid pNN76. The map illustrates the positions of various restriction sites within this fragment. The locations and orientations of the structural genes slt-IIA and slt-IIB are indicated above the restriction map*

Isolation of *slt-II::Tnpho*A gene fusions :

Plasmid NTP707 contains a 4.7 Kb *Eco*RI fragment encoding the entire *slt*-II operon inserted into the chloramphenicol resistance (Cmr) gene of plasmid pACYC184 (Fig. 4.).

Fig 4: Isolation of slt-II::TnphoA Gene Fusions

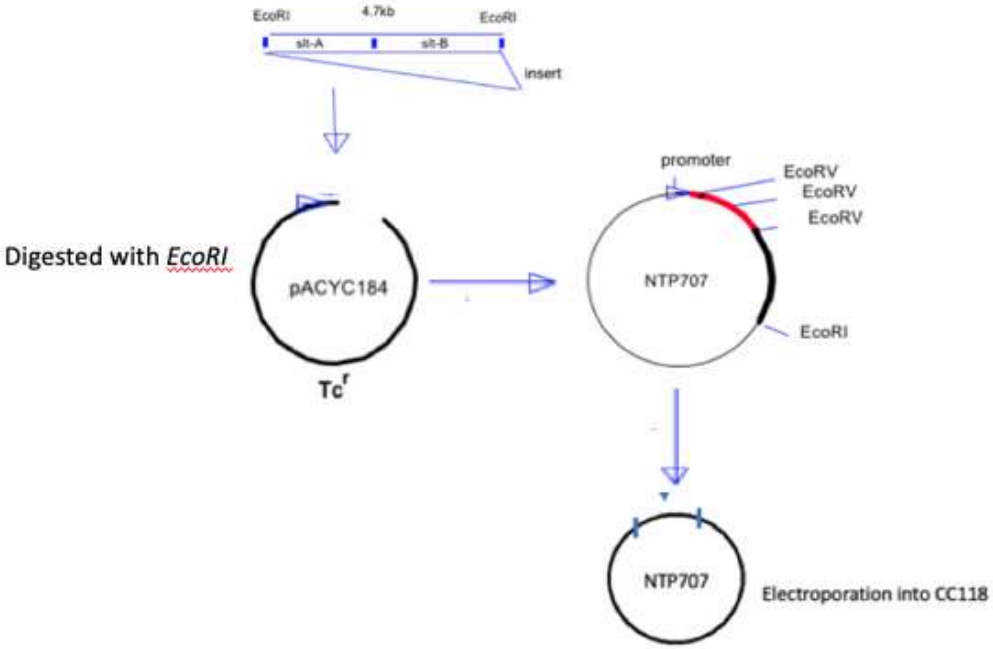

Fig. 4. *Plasmid NTP707 was analyzed to confirm the presence of the slt-II::TnphoA gene fusions. The plasmid contains a 4.7 kb EcoRI fragment that includes the entire slt-II operon. This fragment is inserted into the chloramphenicol resistance (Cmr) gene of the plasmid vector pACYC184. The figure illustrates the location and size of the inserted fragment within the vector.*

E. coli CC118 (table1), which is *Pho*A negative, was electroporated with caesium chloride density gradient purified pNTP707 and the transformants plated onto LB plates containing tetracycline (Tc) to select for pNTP707. To confirm the presence of pNTP707 within *E.coli* CC118, plasmid DNA was prepared from trans- formants displaying tetracycline resistance (Tcr), restricted with *Eco*RI and electrophoresed on 1% (w/v) agarose gels. Two fragments were produced, the first fragment was 4.7 kb, which is the insert containing the slt-II gene and the second fragment was 4.2 kb, which is the vector plasmid pACYC184 (Fig.5: lane 3 and 4). To ensure maintenance of plasmid during preparation, bacteria were cultured on LB containing kanamycin (50 microgram m-1) and tetramycin (15 migram m-1).

Fig. 5: Analysis of pNTP707 in E. coli CC118

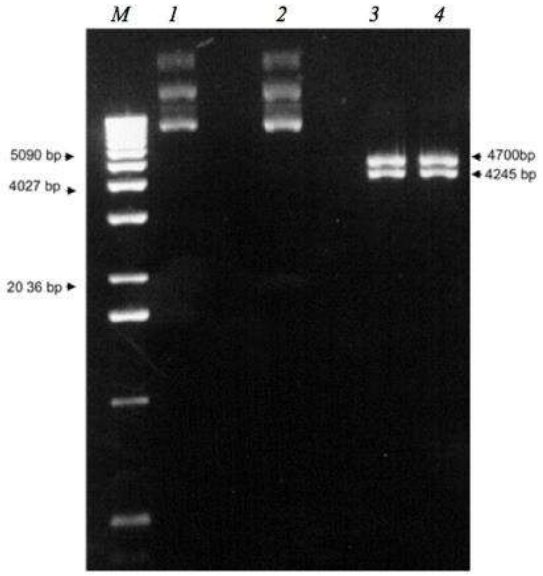

Fig. 5 The gel shows two distinct bands:

Lane 3*: The larger band at 4.7 kb represents the insert containing the slt-II gene.*

Lane 4: *The smaller band at 4.2 kb corresponds to the vector plasmid pACYC184.To maintain the plasmid during the preparation process, the bacteria were cultured in LB medium supplemented with kanamycin (50 µg/mL) and tetracycline (15 µg/mL)*

Plasmid DNA was prepared from transformants displaying Tc resistance (Tcr) restricted with *Sma*l-*Pst*l and electrophoresed on an agarose gel. Restriction with *Sma*l-*Pst*l produced the expected 8 and 0.85 Kb fragments confirming the presence of pNTP707 within *E. coli* CC118 (fig. 6)

Fig. 6. Restriction Analysis of pNTP707

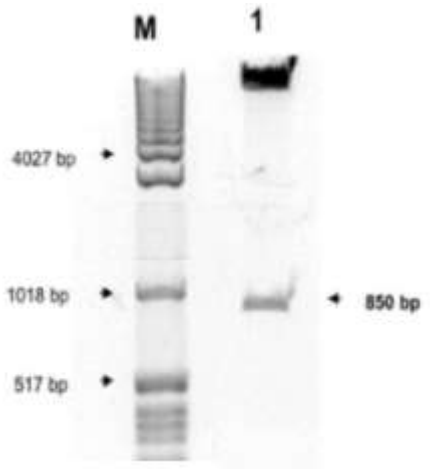

Fig 6. *Plasmid DNA was extracted from E. coli CC118 transformants exhibiting tetracycline resistance (Tcr) and digested with the restriction enzymes Smal and Pstl. The resulting fragments were separated by electrophoresis on an agarose gel. The gel shows two bands: an 8 kb fragment and an 0.85 kb fragment, which correspond to the expected sizes of the digested plasmid pNTP707. This result confirms the presence of pNTP707 within the E. coli CC118 strain.*

Specially designed broad host-range plasmid vectors carrying Tn*pho*A have predominantly been used to deliver *Tnpho*A into target genes of a variety of Gram negative bacteria (Taylor *et al.,* 1987). Typically the broad host-range plasmid vector is introduced into the target organism by conjugation, whereupon transposition of the plasmid encoded *TnphoA,* which encodes kanamycin resistance (Knr), into the chromosome is caused by superinfection with an incompatible plasmid and selection for Knr (Taylor *et al.,* 1987). In the present study, *Tnpho*A was introduced into *E. coli* CC118 (pNTP707) on a defective lambda suicide phage (fig:7and 8). Lambda *Tnpho*A contains a nonsense mutation within a gene essential for bacteriophage replication (Gutierrez *et al.,* 1987).

Fig. 7: Schematic Diagram of Potential Gene Fusion Products from TnphoA Insertion into the slt Operon

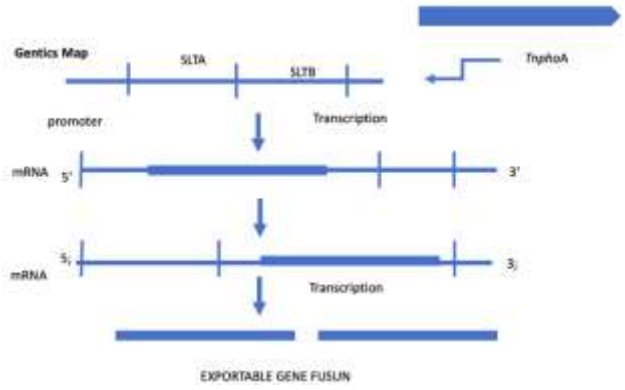

Fig. 7 *This schematic diagram illustrates the potential gene fusion products resulting from the random insertion of TnphoA into the slt operon. The diagram shows the various possible outcomes of this insertion, highlighting how the TnphoA transposon can create different fusion products with the slt operon genes. Each possible fusion product is represented to provide a visual understanding of how TnphoA integration could affect the operon structure and functionality.*

Fig.8: Introduction of TnphoA into E. coli CC118 Using a Defective Lambda Suicide Phage

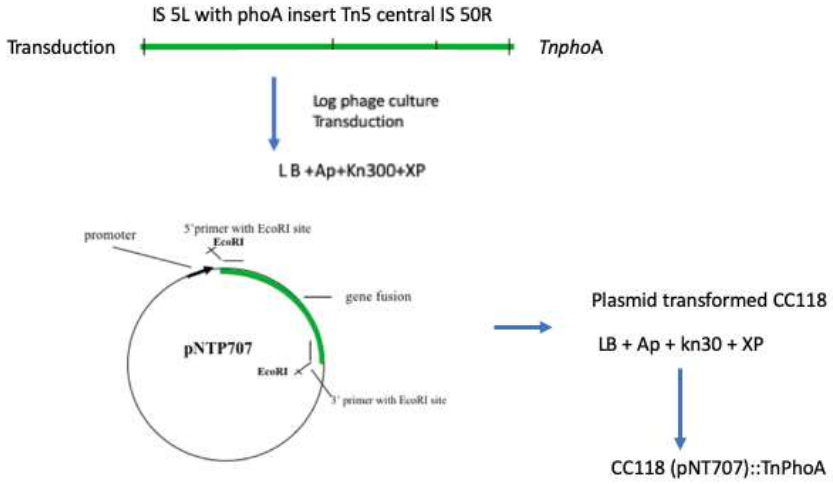

Fig.8. *In this study, TnphoA was introduced into E. coli CC118 carrying plasmid pNTP707 using a defective lambda suicide phage. The lambda TnphoA phage used in this process contains a nonsense mutation within a gene essential for bacteriophage replication, as described by Gutierrez et al. (1987). This modification renders the phage defective for replication, allowing for the stable introduction of TnphoA into the E. coli genome.*

In figure 8 *E.coli* CC118 was electroporated with recombinant plasmid NTP707 and the transformants were selected on LB+ Tc plates and transducted with Lambda TnphoA. Transformants were grown on LB+ Tc + Kn (300)+ XP plates to select for transposition of TnphoA on to the plasmid vector. Fresh CC118 was re-transformed with the plasmid prepared from the blue colonies from LB+ Tc + Kn (30)+ XP plates. These contained in-frame fusions of TnphoA for secreted gene(slt-II) products carried on the plasmid NTP707. *TnphoA* was introduced by transduction of a log phase culture of CC118 (pNTP707) with lambda *TnphoA* (Gutierrez *et al.,* 1987). Growth of transformants on LB+Tc+Kn(300)+XP plates selected for transposition of *TnphoA* onto the high copy number plasmid vector. Such a bacteriophage can propagate in a suppressing (Sup+) host, but is unable to establish itself in a non-supressing (Sup0) host. The high concentration of Kn (300 *pg* ml"*) preferentially selects for transposition of *TnphoA* onto the high copy number plasmid vector (Berg *et al.,* 1983). Growth of transformants on plates containing Kn (300 *pg* ml**) selected for transposition of *TnphoA* onto the high copy number plasmid vector. CC118 was retransformed with plasmid preparations of the transformants and blue colonies on LB +Ap+ Kn(30) + XP

plates isolated. These contained in-frame fusions of *TnphoA* to secreted gene products. The presence and location of *TnphoA* within pSC105 was subsequently confirmed by restriction mapping.

Individual blue colonies on this medium after incubation at 37°C for 2 days, contained in-frame fusions of *TnphoA* to secreted gene products, whereas white colonies represented those strains in which *TnphoA* had not fused to an exported protein, blue colonies grown on LB plates containing Tc, Kn (300 g /ml) and XP and incubated at 37°C for 2 days contained fusions of *slt*::Tn*phoA*, whereas white colonies represented those strains in which Tn*phoA* had not fused to an exported protein (fig. 9)

Figure 9: LB Plates with Competent *E. coli* CC118 Transformed with Plasmid NTP707

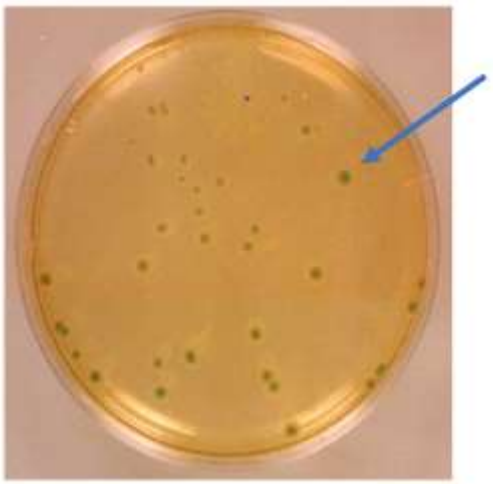

Fig. 9. *LB plates containing fresh competent E.coli CC118 transformed with plasmid NTP707 contain the fusion.*

Individual blue colonies grown on LB plates containing Tc, Kn (300 □g /ml) and XP and incubated at 37°C for 2 days

LB agar plates are shown with fresh competent E. coli CC118 that has been transformed with plasmid NTP707, which contains the fusion construct. The plates demonstrate the presence of colonies, indicating successful transformation and expression of

the fusion gene within the E. coli CC118 cells. To ensure *TnphoA* was not chromosomally encoded, plasmid DNA was prepared from each blue fusion strain on a small scale and used to transform freshly competent CC118. Blue colonies on LB plates containing XP, Tc and Kn (30 *pg* ml'*), contained in-frame fusions of *TnphoA* to secreted gene products on NTP707 (Fig. 10)

Fig. 10: Verification of TnphoA Plasmid Encoding

Fig. 10 .*To ensure that the TnphoA was not chromosomally encoded, plasmid DNA was extracted from each blue fusion strain on a small scale and used to transform freshly competent E. coli CC118 cells. The transformed cells were plated on LB agar supplemented with X-Phosphate (XP), Tetracycline (Tc, 30 µg/ml), and Kanamycin (Kn, 30 µg/ml). The appearance of blue colonies on these plates indicates that TnphoA is present as an in-frame fusion with secreted gene products on the plasmid NTP707, confirming that the fusions are plasmid-borne rather than chromosomally integrated.*

The presence and location of *TnphoA* within plasmids was then confirmed by restriction mapping. As stated earlier, *TnphoA* must insert itself in the correct orientation and reading frame to create an active fusion. Gott & Boos (1988), however, have shown *TnphoA* to be capable of generating active fusions, even when inserted out of frame by +1. This is thought to be due to *in vivo* frameshifting where the translational machinery corrects the wrong frame by reading a sequence of four nucleotides as a sense codon.

Restriction mapping of *slt-TnphoA* fusions:

Fig11. Restriction Mapping of slt-TnphoA Fusions

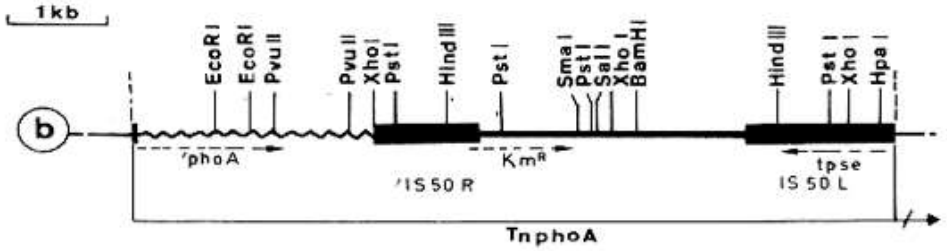

Fig.*11. This figure illustrates the restriction mapping of slt-TnphoA fusions. The map shows the positions of various restriction enzyme sites within the fusion constructs. The fusion junctions between the slt gene sequences and the TnphoA transposon are indicated, highlighting the integration points and providing insight into the genetic organization of the fusions. This mapping is crucial for characterizing the structure and orientation of the fusions in the context of the slt-TnphoA constructs. The structure of TnphoA. with orientation of the phoA fragment indicated. No sites : EcoRV, SacI, XbaI, PvuI. One site :DraIHpaI, BamHI, SalI.*

Fig. 12. Simplified Map of Transposon TnphoA (Manoil and Beckwith, 1985)

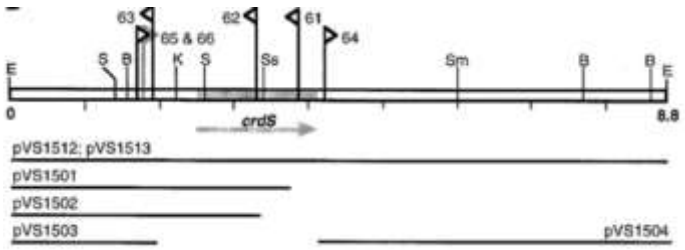

Fig.*12. This figure provides a simplified map of the TnphoA transposon, highlighting key features of its structure. The map shows the locations of the insertion sequence IS50L, which contains the* phoA *gene, the* kan *gene, IS50R, and various restriction enzyme sites. The layout of these components is depicted to illustrate the arrangement and orientation of the genetic elements within the transposon.*

After *Tnpho*A was introduced into E. coli CC118 (pNTP707), plasmid DNA was prepared from the blue colonies and restricted with EcoRV. This enzyme cuts the plasmid at one site in the vector plasmid pACYC184 and at three sites in the 4.7 kb EcoRI

insert. TnphoA has no EcoRV restriction sites. Plasmids were prepared from bacterial strains by small scale alkaline lysis, and digested with the enzyme EcoRV. This restriction endonuclease cuts plasmid NTP707 at one site in the vector plasmid pACYC184 , and three sites in the 4.7 Kb *EcoRI* insert (figure 13). Transposon TnphoA has no EcoRV restriction enzyme sites. Thus EcoRV digestion of plasmid NTP707, without a TnphoA insert, gives rise to four fragments of approximately 0.9, 1.3, 3.0, and 3.8 Kb (tracks 2 figure 13).

fig.13. *EcoR*V Digestion of Plasmid NTP707 with and without TnphoA Insert

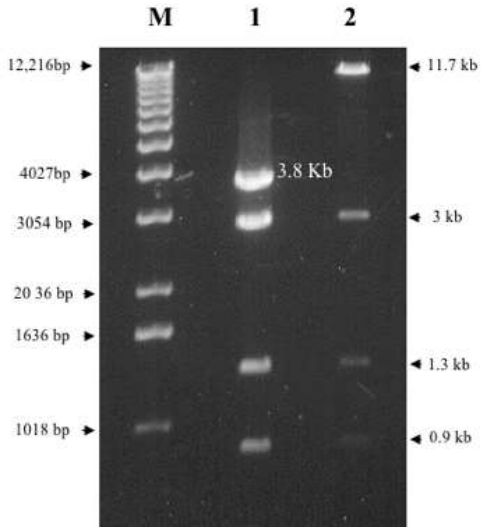

Fig 13. *This figure demonstrates that the transposon TnphoA lacks EcoRV restriction enzyme sites. Consequently, digestion of plasmid NTP707 (which does not contain a TnphoA insert) with EcoRV yields four distinct DNA fragments of approximately 0.9 kb, 1.3 kb, 3.0 kb, and 3.8 kb. These fragment sizes are shown in tracks 2 and 3 of the gel image in Figure 8. The absence of TnphoA insert results in the predictable pattern of fragments, serving as a control to confirm the integrity of the plasmid and the absence of the TnphoA sequence.*

The fusion of *TnphoA* to the *slt-l*A subunit gene (Calderwood & Mekalanos, 1987), and to the s/f-IIB subunit gene (this study) allows measurement of expression of the *slt-l* and *sti-U* operons under different growth conditions by assay of PhoA activity. Although many studies have involved the use of multicopy fusion plasmids, plasmid copy number has been shown to vary considerably with such parameters as promoter strength and cellular growth conditions (Adams & Hatfield, 1984). It is therefore desirable to have single copy derivatives of the *slt-l::TnphoA* and *slt-U::TnphoA* fusions to eliminate the problems associated with uncontrolled fluctuations in plasmid copy number which affect gene dosage.

Dissection

Fusions to both a secreted periplasmic protein and a complex cytoplasmic membrane protein led to alkaline phosphatase activity. TnphoA fusions should help localize export signals within the structure of a protein, such as a transmembrane

protein, as well as identify new chromosomal genes for secreted and transmembrane proteins. The *Tnpho* A transposon inserts into the genome through a process called transposition. This involves the transposase enzyme recognizing and binding to specific sequences flanking the transposon, facilitating its integration into a new location in the host DNA. Once inserted, the *Tnpho* A gene can create a fusion protein by linking the *Pho*A enzyme with a target protein or gene of interest. This fusion protein can then be expressed and analyzed to understand the function and location of the target protein within the host cell. A single copy *slt::TnphoA* gene fusion in the wild type slt-II-producing strain was subsequently developed. Use of the latter assay system in association with the *slt::TnhoA* gene fusion on the recombinant plasmid pSC105 (Calderwood & Mekalanos, 1987), enabled specific quantification of slt I and slt-II expression during growth under aerobic, anaerobic and iron limiting conditions . The formation of SLT II (Shiga-like toxin II) through Tnpho A gene fusion is a fascinating topic in microbiology and genetic engineering. This discussion will explore the mechanisms, implications, and applications of this genetic fusion, focusing on the scientific and practical aspects. The Tnpho A gene fusion involves the integration of the Tnpho A transposon into the genetic material of a host organism. Tnpho A is a type of transposon that carries a gene encoding a fusion protein. This fusion protein typically includes a reporter enzyme like alkaline phosphatase (PhoA), which is used to study gene expression and protein localization. The fusion of SLT II with Tnpho A allows researchers to study the expression, localization, and activity of Shiga-like toxin II within cells. SLT II is a potent toxin produced by certain bacteria, and understanding its behavior in a cellular context can provide insights into its pathogenic mechanisms. The use of Tnpho A as a reporter system is particularly valuable. Alkaline phosphatase is an enzyme that can be easily detected through colorimetric assays, making it an excellent tool for monitoring gene expression and protein interactions. Insights gained from studying SLT II through Tnpho A fusions might inform the development of novel therapeutic strategies to counteract the effects of Shiga-like toxins. This could include designing inhibitors that block toxin activity or developing vaccines that target the toxin. SLT II, like its counterpart SLT I, is associated with severe illnesses such as hemolytic uremic syndrome (HUS). By studying SLT II's activity and its interactions with host cellular components through Tnpho A fusion, researchers can gain a deeper understanding of the toxin's role in disease processes. The *Tnpho* A gene fusion technique could be adapted for diagnostic purposes, helping to identify and characterize bacterial strains that produce SLT II. This could lead to more rapid and accurate diagnostic tools for diseases associated with this toxin. The principles of gene fusion and reporter systems can be applied in biotechnology to create novel proteins with desirable properties. By engineering fusion proteins, researchers can create tools for a variety of applications, from industrial processes to research reagents. Investigations of the regulation of the bacteriophage-encoded Shiga-like toxin II (SLT-II) in *Escherichia coli* demonstrated that bacteriophages exhibit a regulatory impact on toxin production by two mechanisms. Firstly, replication of the toxin-converting bacteriophages brings about an increase in toxin production due to concomitant multiplication of toxin gene copies. Secondly, an influence of a phage-encoded regulatory molecule was demonstrated by using low-copy-number plasmid pADR-28, carrying a translational gene fusion between the promoter and proximal portion of slt-IIA and the structural gene for bacterial alkaline phosphatase (phoA). PhoA activity, reflecting the slt-II promoter activity, was significantly enhanced in *E. coli* strains which and been lysogenized with an SLT-I or SLT-II-converting bacteriophage (H-19B or 933W, respectively) or bacteriophage lambda. The inactivity of cytoplasmic alkaline phosphatase appears to be due, at least in part, to the absence of essential intrachain disulfide bonds (J. Beckwith, unpublished results).

CONCLOSION

In the formation of *a slt::TnphoA* gene fusion , both the A and B subunits are synthesised with amino terminal signal sequences. *TnphoA* can therefore fuse into either subunit gene and be exported into the periplasm to give enzyme activity. As both genes are transcribed from a single promoter upstream of the A subunit gene in *slt*-II both possible gene fusions allow monitoring of *slt*-II gene expression to be undertaken. The formation of *SLT* II through *Tnpho* A gene fusion represents a powerful approach to studying and manipulating bacterial toxins. The use of transposon-based reporter systems facilitates detailed investigations into protein function and interaction, offering valuable insights into pathogenic mechanisms and potential therapeutic targets. As research progresses, the applications of these techniques are likely to expand, contributing to advancements in diagnostics, therapeutics, and biotechnology.

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