Tracking Autotransporter Secretion with Fluorescent Tags

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Declaration: I declare that all the work presented in this dissertation is my own, except where otherwise stated.

Date: August 2007

Signature: .....

Autotransporters (ATs) are a large family of virulence associated proteins secreted from Gram-negative bacteria and those ATs studied to date play an important role in pathogenisity and disease formation. Recently, monitoring of such protein secretion steps by using fluorescently labelled proteins becomes a common practise. We have successfully inserted the mcherry protein derived from the indo-pacific sea mushroom *Anemone Discosoma Striata* into the EspC AT derived from *Enteropathogenic E. coli*. However, the green fluorescent protein (GFP) from the jellyfish *Aequoria vicroria* insertion was unsuccessful as it requires TAT system for its proper folding in the periplasm. We demonstrated that the EspC-mcherry construct is localised at polar and multiple sites over the bacterial surface during its expression in the wild type (MG1655) and mutant strains *(Δnei, ΔproW, ΔsapA, ΔybgC, ΔslyB, ΔabrB and ΔygjE).* Western blot and SDS PAGE analysis reveal that whole length EspC-mcherry 174-kDa protein was presented and localised at the outer membrane in all the strains, consistent with protein secretion. In addition, some mutant strains exhibited a 70.5-kDa mcherry containing protein, while in others it was almost absence, suggesting that some mutants may show a protein localisation defect. It was predicted that the 70.5-kDa protein lacks its β-barrel domain and therefore should not be found in the outer membrane fraction where its localisation was confirmed by using exogenous trypsin digestion. Further analysis is required to determine whether EspC-mcherry or any of its derivatives form aggregates that have surreptitiously co-localised with the outer membrane during the analyses performed in this study.

Autotransporters (ATs) are virulence associated proteins which play an important role in pathogenicity in the Gram-negative bacteria (1). Pohlner et al. were the first to identify and describe the type V secretion pathway, which is used by ATs to exit the cell. To explain the extracelluar location of immunoglobulin A1 protease secreted by *Nesseria gonorrhoeae*, they concluded that the IgA1 protease precursor has 2 functional domains: an amino terminal domain (N-domain) known as the "leader" was proposed to initiate inner membrane translocation of the precursor via the Sec machinery resulting in a periplasmic intermediate, while a carboxy terminal "helper" domain is important for outer membrane secretion. Furthermore, they proposed that protein transport through the outer membrane is mediated entirely by its carboxy terminal domain, and that no energy coupling or accessory proteins are needed to perform the translocation process. Moreover, Pohlner et al. were the first to assume that the carboxy terminal part of IgA precursor might form an integral membrane structure analogous to that of porins, constructed from antiparallel β-sheets organised in a hypothetical β-barrel conformation to permit the amino terminal of the AT to pass through a β-barrel pore to the bacterial surface (2). Since that explanation, a large number of proteins which are secreted by an analoguous mechanism have been discovered among Gram-negative bacteria (3). In fact, all AT molecules contain 3 distinct domains: the signal sequence that is located at N-terminal end of the proteins. The following domain called the passenger domain (also called α- domain, translocation unit or N-domain) which encodes diverse functions such as proteolytic degradation. The last domain, known as the carboxy domain (β-domain or transporter domain), contains a specialised unit called the translocation unit which plays a significant role in autotransporter secretion across outer membrane (4, 5).

To enable inner membrane transport, the N-terminal signal peptide of ATs is recognised by the cytoplasmic chaperone SecB "post-translocational secretion" (5, 6) or by the Signal Recognition Particle (SRP) "co-translocational secretion" for example Hbp (7, 8). The structure of signal sequences is well understood, they have n1 and n2-domains with positive charged amino acids, h1 and h2-domains containing hydrophobic amino acids and a c-domain incorporating a proteolytic recognition site that usually comprises of proline and glycine residues and uncharged short lateral-chain residues which determine the site of signal sequence cleavage (9). Intrestingly, some ATs have unusual long signal sequences with an extension in the n-domain (5). Such ATs bearing extended signal peptides have been shown to have unusually slow translocation across the inner membrane e.g. EspP (10).

The content within n1 and h1 domains are quite unusual because of the presence of aromatic amino acids in the n1 domain, and a glutamate residue in the h1 domain, this conservation in sequence indicates existence of specific functions, suggesting that such extension in signal sequence has a role in direction of protein secretion across the inner membrane through a pathway different from the conventional Sec pathway, such a pathway may employ SRP instead of SecB, or an accessory factor that is currently unidentified (5).

At the inner membrane, the AT docks with the ATPase SecA initiating translocation (6). After passage of the AT carboxy terminal through the SecYEG pore, which is located in the inner membrane with facilitator accessory proteins such as SecD, SecF and YajC, signal peptides are cleaved by membrane bound leader peptidase allowing release of the proprotein into the periplasmic space (11). The existence of a periplasmic intermediate has been supported by a study using a reporter antibody as a reporter passenger domain to investigate the process of secretion. In this study it was concluded that folding of the passenger domain occurs in the periplasm before it is exposed outside (12). In contrast, an investigation by Oliver et al. revealed that folding occurs on the bacterial surface rather then in the periplasm (13). Another investigation has demonstrated that secretion of an AT across the outer membrane is dependent on the folding of protein domains containing disulphide bonds, and on the need for partial unfolding for efficient secretion. To accommodate this, passenger domains would be exposed to the action of chaperones such as FkpA and DsbA during their periplasmic transit to assist the ATs folding, disulphide bond formation and preventing aggregation (14). This controversy illustrates that the nature of periplasmic ATs remains unclear, and that more investigation are needed (11).

The C-terminal transporter domain of ATs responsible for passenger domain secretion across the outer membrane contains 10-14 β-strands. These β-strands are connected with each other by hairpin turns and loops forming an anti-parallel- β -barrel. This is connected to an α-helical region known as the "linker". It has been assumed that the linker forms a hairpin structure embedded in the outer membrane channel, allowing pulling of the N-terminal passenger part to bacterial surface (15). Furthermore, it has been suggested that the outer membrane accessory factor Omp85 plays an important role in β-barrel sheet formation (16). It has also been proposed that β-domains of the ATs can form an oligomeric ring-like complex with a ~2nm central hydrophilic channel through which export of the folded protein domains might occur. This has led to the introduction of a new AT secretion model. Unfortunately, this model has not been accepted due to the thought that ATs must be unfolded during their export across outer membrane (17). Once the ATs are located at the surface of the bacteria, the passenger domains are exposed to alternative fates: first, it may be cleaved from the C-terminal and released into the extracellular space by an autoproteolytic mechanism or by the action of proteases found on the cell surface. Second, it might be cleaved but still non-covalently linked with the C-terminal. Lastly, it remains covalently attached to the β-domain and still permanently on the cell surface (11). Although some of the key features of AT transport have been identified, the precise mechanism is uncertain and more studies are required to understand it.

Fluorescent proteins are increasingly being used as a reporter or as a monitor for protein production, secretion, purification, folding and cellular responses in both prokaryotes and eukaryotes (18). They are tagged to the protein of interest by fusion. However, such fusion can cause disturbance in function of the target protein. Wild type fluorescent proteins are often obligatory tetramer, disruptive and toxic (23), so, since these genetically encoded fluorescent tags are a very attractive tool to monitor bacterial cell biology, they have been improved. The Green Fluorescent Protein (GFP) derived from the Jellyfish *Aequoria Victoria* (19) has several properties which make it one of the best fluorescent proteins, it is attractive because of its self contained fluorescence mechanism and as no co-factors are required for its fluorescence (20). Furthermore, its red-shift can be obtained by blue light excitation with less UV-induced toxicity and photobleaching (21). Moreover, its stability during tagging and protein purification as well as indirect immunofluorescent protocols permits observance of its fluorescence *in vitro*. Finally, new technological protocols have been discovered as a result of multiple features that GFP has, such as Resonance

Energy Transfer (FRET) for protein-protein interaction studies (21). To enhance the usefulness of GFP, variants have been generated by mutagenesis and genetic manipulation of the *jellyfish* GFP. These new variants such as a green colour variant of *Aequoria* GFP (EGFP), a cyan fluorescent variant (ECFP), and the yellowish-green fluorescent protein (EYFP) are distinct from each other. For instance, EGFP has high quantum yield and resistance to photobleaching, whereas EYFP has the highest absorption and quantum yield but is more vulnerable to photobeaching (22). In addition to the wild type GFP and its variants, large numbers of other fluorescent proteins have been innovated that display a variety of spectral features. These include red fluorescent proteins (dsRed) derived from *Anemone Discosoma Striata* (22) that share only 25% of similarity in sequence with GFP (22). One major advance was the evolution of a monomeric fluorescent protein from dsRed called mRFP1 (23, 24). MRFP1 has been exposed to several rounds of focussed evolution by using fluorescence activated cell sorting (FACS) based screening. This has led to innovative new deviations characterised by several new colours, enhanced stability of N- and C-terminal fusions and enhancements in photobleaching, quantum yield and extinction coefficients. The culmination of these studies is mcherry (23).

Recently, the use of fluorescent proteins for monitoring secretion has become a common practise. For instance, GFP has been translationally fused to different parts of the AT IcsA in order to define where ATs are secreted and the region in IcsA responsible for its secretion toward the bacterial old pole (25). Furthermore, those constructs have also been used to investigate IcsA localisation in spherical cells (26). Another investigation demonstrated that localisation of AT proteins at the pole of bacteria occurs in the cytoplasm and is independent of the secretion pathway. This latter study did not involve fusion of GFP to IcsA and SepA ATs from *shigella flexneri*. Rather, localisation was monitored by using antibodies against IcsA and SepA and thus is an example of an approach using indirect fluorescence microscopy (27). Another study used GFP fused to the cytoplasmic membrane protein YidC, which is needed for membrane insertion of a number of SRP-secreted proteins. Such fusion led to increased fluorescence at both bacterial poles, suggesting protein secretion that is dependent on YidC might occur at bacterial poles (28).

The aim of this project is to track and monitor the secretion stages of an AT protein derived from *Enteropathogenic E. coli* (EPEC) called EspC (29, 30). This will be achieved by fusing fluorescent tags into EspC in order to track protein secretion at different stages and to study protein folding, solubility and stability of EspC and obtain active recombinant proteins with high yield and purity (18). Two fluorescent tags will be used: GFP and mcherry. Moreover, the project will identify the specific points in the cell where the fusion protein accumulates using fluorescent microscopy. Finally, monitoring secretion in a set of *E.coli* MG1655 mutants defective for EspC secretion will reveal whether the accessory factors are essential for secretion.

EXPERMENTAL PROCEDURES

Bacterial strains, Plasmids and Growth culture— The bacteria and plasmids used in this study are listed in table 1. *E coli* MG1655 strains was mutated in the genes (*nei*, *proW*, *sapA*, *ybgC*, *slyB*, *abrB* and *ygjE*) which are thought to be involved in the AT secretion. These genes were identified using transposing mutagenesis and were screened for deficient EspC secretion by Louise Arnold. Newly constructed mutants have been used in this experiment, these were made by Dr Stephanie Pommier in the same genes via the Datsenko and Wanner method, which introduced a clean deletion (31). All bacterial strains were grown on Luria-Bertani (LB) agar or liquid shaking at 37˚C with ampicillin (100µl/ml) as needed. Expression of arabinose promoters was induced with 0.2% w/v arabinose (final concentration), whereas expression of *lac* promoter was induced by addition of 10mM IPTG (final concentration).

Primer design and PCR—Primers were designed using clustal W, reverse complement and promega websites and have been manufactured by Sigma. The restriction enzyme *BglII* (from *BioLabs*) has been used in this study and the reason is that it cuts just once in the middle of *espC* (in passenger domain) without affecting the

TABLE 1: **Bacterial Strains and Plasmids used in this study.**

Strains or Plasmid	Genotype or Description	Reference or Source
MG1655	*E. coli* K12, F-lambda-*ilva-rfb*-50-*rph-1*. *supE4* Δ*lacU169* (Φ80 *lacZΔM15*) *hsdR17*	*E coli* Genome project www.genome.wise.edu
DH5α	*recA1 endA1 gyrA96 thi-1 relA1*.	32
MG1655 Δ*sapA* MG1655 Δ*slyB* MG1655 Δ*ygjE* MG1655 Δ*nei* MG1655 Δ*proW* MG1655 Δ*ybgC* MG1655 Δ*abrB*	MG1655 with deletion of accessory genes using Datsenko and Wanner method.	Dr Stephanie Pommier
pladc1	pBad18 containing espC gene under arabinose promoter plus Ampicillin resistance gene.	Sue Dodson
pEGFP	Plasmid containing Green Fluorescent Protein (GFP).	Clontech*
Pmcherry	Plasmid containing fluorescent protein mcherry.	23
pmac	pladC1 Plus mcherry insert and Ampicillin resistance gene.	This study

- www.clontech.com/images/pt/dis_vectors/PT3078-5.pdf

plasmid that is carrying it. Primer sequences are shown in table 2. Primers need to be designed such that one recognises the sense strand of DNA (same sequence as antisense strand) while the other recognises the antisense strand of DNA (same sequence as sense strand). Primers contain 18 nucleotides complementary to the 5` end of the gene of interest plus the *BglII* restriction site 5`-AGATCT-3` and a thymine nucleotide between the ATG and *BglII* restriction site to keep the sequence in frame (table 2). The same steps were applied to the 3` end of the gene, except that two thymine residues were added to maintain the reading frame.

TABLE 2: **Primer Sequences.**

Primer Name	Primer Sequence (5`to 3`)
GFP1	***CT***AGATCT**T**ATGGTGAGCAAGGGCGAG
GFP2	***AG***AGATCT**AA**CTTGTACAGCTCGTCCAT
Cherry1	***CT***AGATCT**T**ATGGTGAGCAAGGGCGAG
Cherry2	***AG***AGATCT**AA**ACTTGTACAGCTCGTCCA

Underlined nucleotides = restriction sites for *Bgl II* enzyme.
Nucleotides in blue bolds = additional bases to bring in frame.
Nucleotides in red bolds and italics = additional bases to give efficient cleavage.

In addition, cytosine and thymine were added at the end of the primers to encourage efficient cleavage by *BglII* (table 2). Finally, the reverse complement website was used to provide the sequence of the second primer. With regard to PCR, each reaction was performed in a total of 30µl comprising: 1-2 µl pEGFP or pmcherry template DNA, 3µl from stock concentration 100µM of each primer, 3µl from 2.5mM of dNTPs, 1µl of 3.5U/µl of Taq DNA polymerase (from *Roche*), 3µl of 10X buffer 2 (from *Roche*) and 16µl sterile water. PCR was performed using the following conditions; denature at 95˚C for 5 min, 30 sec at 95˚C, anneal at 55˚C for 30 sec and extend at 72˚C for one

min. The final elongation step was at 72˚C for 10 min. The cycle was repeated 30 times and samples were held at 4˚C. 6µl of orange buffer containing 40% sucrose and 0.4% orange dye in TAE buffer 6X was added to each PCR product, whereas 5µl from same buffer was added to 7µl of DNA ladder (promega 1kb DNA ladder) and the products were separated on a 0.8% agarose gel.

DNA Manipulation and cloning — DNA was manipulated by standard methods (33). Extraction of plasmid DNA from agarose gel was preformed by using QIA quick Gel Extraction Kit, whereas plasmid DNA preparation was prepared by using QIA prep Spin Miniprep Kit. After extraction of PCR products contain *egfp* and *mcherry*, digestion was performed in a total volume of 40µl, whereas the digestion of the pladc1 vector was performed in 60µl volume for 6hrs at 37˚C. The digestion included *BglII* and 1X buffer3 (from *BioLabs*). Following digestion, ligation of the digested PCR products into pladc1 vector took place in a total volume of 20µl where T4 DNA ligase (from *BioLabs*) and its buffer were used. Moreover, controls were set up in this step which contained just pladc1, ligase and buffer without the digested PCR product. In fact, antarctic phosphatise (from *BioLabs*) has been used to treat pladc1 vector (1µl of 10X antarctic phosphatise buffer + 1µl of 5U/µl of antarctic phosphatise + 10µl pladc1, 15 min at 37˚C then 5 min at 65˚C) before setting the ligation and the purpose of that is to prevent the plasmid self ligating. It works by removing the 5` phosphate group on both vector termini. With regard to transferring the plasmid into *E. coli*, competent cells were prepared using 50mM $CaCl_2$ glycerol 15%, heat shock has been chosen in this study and the procedure of such transformation is 45min on ice, 2 min at 42˚C, 5 min on ice, 10 min at room temperature 20˚C then 800µl of fresh LB added followed by incubation at 37˚C (shaking) and centrifugation 3 min at 6000 rpm. Lastly, approximately 500µl of supernatant was discarded before resuspending the pellet and spreading them on Amp plates incubated overnight at 37˚C.

SDS PAGE, Western Blotting and Subcellular Fractionation—Proteins were separated by SDS PAGE and stained with coomassie blue as described previously (34). Similarly, western blotting was preformed as described (34). Anti-dsRed (from *Clontech*) has been used as primary antibody at a dilution of 1:1000, whereas the second antibody, anti-rabbit IgG peroxidase (from *Sigma*), has been used at a dilution of 1:2000. Fractionation was made by using osmotic shock as described (35), spheroplast preparation as explained in (36) and Cambridge protocol as clarified in (37). Trichloroacetic Acid (TCA) precipitation of the supernatant was carried out as described (38). Samples were sonicated for 10 sec and boiled for 5 min at 95˚C after resuspending in 1X SDS PAGE loading buffer. Trypsin digest was also applied to some samples by dilution of 100µl of the overnight culture into 10 ml of fresh LB containing 10µl ampicillin, shaking at 37˚C for 2hrs. Subsequently, induction was made by adding 100µl of 0.2% arabinose, shaking at 37˚C for 3hrs. After reaching OD_{600} = 6, pelleted cells were resuspending in 600 µl of 100mM Tris-HCL pH 8.0 and then the samples were divided into two volumes (i) 100 µl as a control and (ii) 500 µl add to it 50 µl of 1mg trypsin in 500 µl100mM Tris-HCL pH 8.0, and kept shacking at 37˚C for 60 min. Finally, samples were centrifuged at top speed for 2 min and pellet resuspended in 100µl of 1X SDS PAGE buffer for western blot analysis.

Wallac Multilabel Counter 1420 and Nikon Fluorescent Microscopy—Interesting colonies have been cultured on LB plates containing ampicillin, where each colony has been applied inside a numbered square using a template. After incubation overnight at 37˚C, colonies from each numbered culture were inoculated into individual wells of a 96 well plate containing 250 µl of fresh LB, 0.2µl ampicillin and 0.2% arabinose. The plate was then incubated overnight at 37˚C shacking before being read by Wallac Multilabel Counter. With regard to fluorescent microscopy, cell culture is prepared by picking up a target colony and inoculating into 2ml of fresh LB and 2µl of ampicillin, and then the culture is left to grow at 37˚C shaking for 2 hrs. Induction for 3 hrs takes place by adding 20µl of 20% w/v arabinose shacking at 37˚C. In addition, slides overlaid with agar were prepared by mixing 90mg oxoid bacteriological agar in 6ml buffer containing 60µl 1M Tris, 60µl $MgCl_2$ pH 7.4 and 5.880 ml sterile water to give 1.5% final concentration. 300µl of this mixture was placed onto each a glass slide, a cover slip was applied and the agar left to

set. 8µl of each bacterial culture was applied to the solidified agar after removing the cover slip and then the agar was covered with a new cover slip before being observed under Nikon Japan microscope fitted with a fluorescent attachment. GFP and mcherry were visualized under illumination with blue and green light respectively, and pictures were taken by using Nikon Digital Camera DXM1200 connected to Nikon ACT-1 software.

RESULTS

Construction of a fluorescent AT—The first objective of this project was to tag fluorescent proteins (GFP or mcherry) into the EspC AT without disturbing the AT function (Fig. 1). Towards this aim, PCR amplified DNA encoding the fluorescent proteins (Fig. 2A) and the pladc1 vector (encoding EspC) were digested with restriction enzymes (Fig. 2B) ligated together, and then transformation into *E. coli* strain DH5α. This cloning strategy was subjected to a number of optimisation steps which included (i) altering the concentration of the pladc1 vector by digesting it in either a 20µl or 60µl volume, (ii) changing the volume of the ligation (either 10µl or 20µl), and (iii) subjecting the pladc1 vector to treatment with antarctic phosphatase prior to ligation to prevent self-ligation. Combining the pladc1 digested in the largest volume with the largest ligation volume (cloning 1, Fig. 2A-C) yielded a reasonable number of transformants on the ampicillin containing selection plates after incubation overnight at 37˚C; 50 colonies potentially with a GFP encoding insert, 46 colonies with mcherry and around 50 colonies on the control plate transformed with a ligation mix lacking insert DNA encoding a fluorescent protein. Since the high number of colonies on the control plate indicated the large proportion of self ligating pladc1 plasmids under these conditions, this plasmid vector was treated with phosphatise and the cloning steps repeated (cloning 2, Fig. 2D-F). This time:

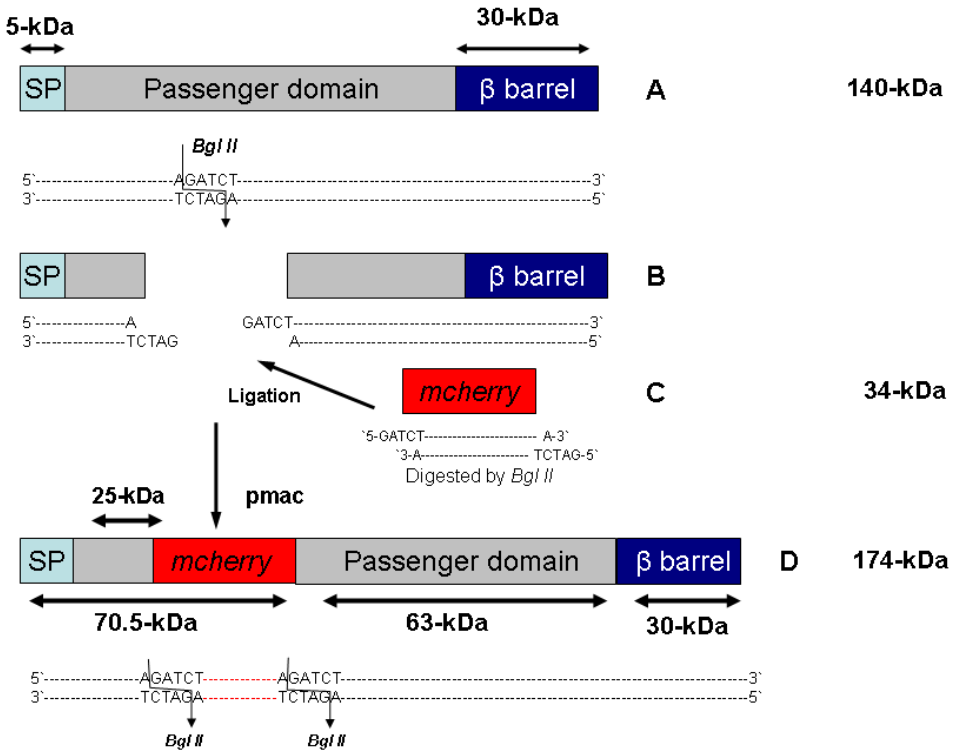


FIG. 1: Cloning Strategy, insertion of mcherry protein into EspC AT. **A**, shows EspC AT protein, its size between 110 to 140-kDa, and the restriction site for *BglII* enzyme on its DNA template that employed by pladc1 plasmid. **B,** sufficient cleavage of the DNA template by *BglII* enzyme resulting in a protein cleavage then ligation take place by insert the digested mcherry protein by the same enzyme into the cleavage site. **D**, successful insertion of the fluorescent mcherry protein into passenger domain result in pmac formation.

18 colonies were observed with the control pladc1 only ligation, 4 colonies resulted from ligation of mcherry with untreated pladc1, and 14 colonies from ligation of mcherry with treated pladc1.

To detect which of these colonies is fluorescent and thus confirm successful cloning, the transformats were inoculated into numbered squares on an ampiciliin plate, and left to grow overnight at 37˚C. Subsequently, each was individually inoculated into two wells of a 96-well microtitre plate (one containing LB, and the other LB plus arabinose to induce *espC* expression), and left to grow overnight at 37˚C. Wallac Multilable Counter 1420 was used to detect the light-emitting and light-absorbing wavelength. One colony with mcherry (No 75) was interesting as it recorded a peak of light emitision, whereas two from GFP colonies were quite interesting (No 34 and 59). In order to investigate whether these interesting colonies contained the correct mcherry or GFP inserts, a plasmid miniprep was performed followed by digestion and separation through a 0.8% agarose gel. It was discovered that the colony number 75 from the first cloning contained the expected insert (Fig. 1C), and the plasmid it contained was named pmac75. From the second cloning with phosphatise treated pladc1, 4 plasmids containing mcherry were successfully constructed (pmac 1,9,10 and 12) (Fig. 1F). No inserts were observed containing the expected GFP insert leading to the conclusion that the GFP cloning was unsuccessful (Fig. 1C).

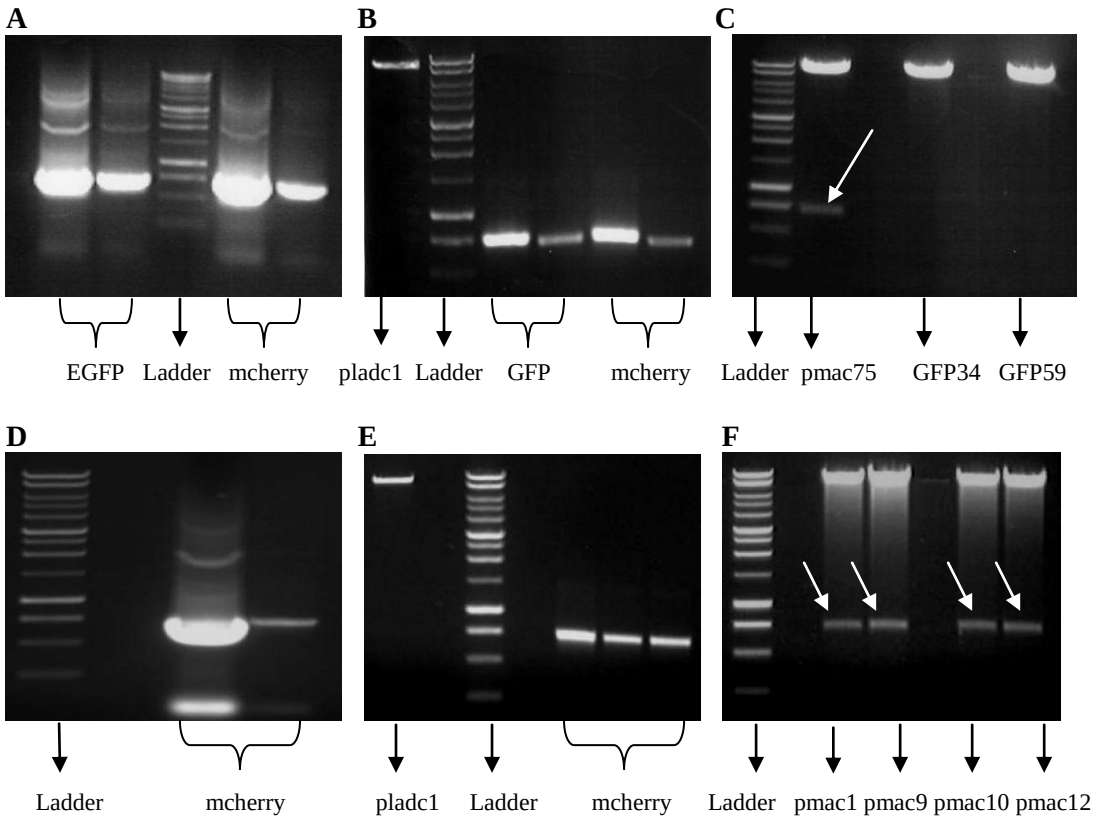


FIG. 2. mcherry is successfully inserted in to pladc1. **A**-**C**, first cloning. **D**-**F**, second cloning. **A** and **D** show the PCR products of both GFP and mcherry. Each was applied to two adjacent lanes of the agarose gel on either side of the 1kb DNA ladder. **B** and **E** illustrate the digested genes and pladc1 vector. A portion of the *Bgl*II digested pladC1 or PCR product bearing the gene encoding either GFP or mcherry as indicated was applied to an agarose gel. **C** and **F** confirm the successful cloning by observing the expected sized inserts (indicated by the white arrows) on an agarose gel following digestion of plasmids in fluorescent transformants with *Bgl*II.

Fluorescent microscopy reveals polar localisation of the EspC AT— In order to monitor the secretion of EspC AT containing mcherry, 2µl of the newly constructed plasmids (pmac75 or pmac9) were transformed into *E. coli* strain

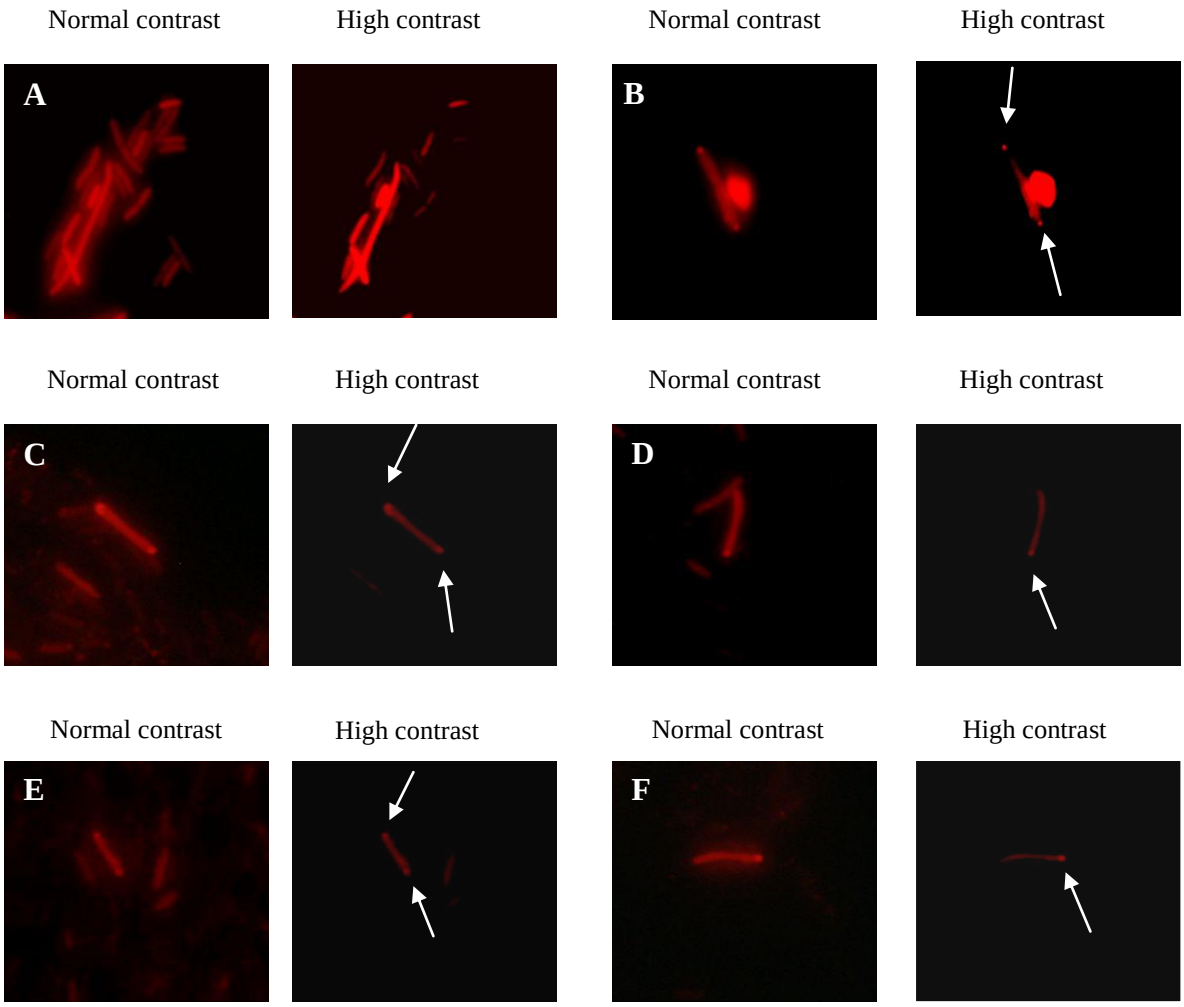


FIG. 3: Microscope images of *E. coli* cells tagged with mcherry, induced with arabinose and illuminated by green light. **A,** the control, mcherry in DH5α. **B,** pmac75 in MG1655, polar localization of EspC secretions (white arrows). **C, D, E and F,** pmac9 in MG1655, polar localization of EspC secretions (white arrows).

MG1655. Furthermore, for comparison pmcherry was transformed into *E. coli* strain DH5α. Subsequently, after cell culture dilution and induction, cell suspensions were placed on the agar coated slides in order to immobilise the bacteria for the accurate capture of phase contrast and fluorescent images since cell mobility was detrimental to this. All cells displayed red fluorescence under green light exposure. Moreover, in contrast to the control cells (producing mcherry diffusely in the cytoplasm, Fig. 3A), those producing mcherry fused to EspC from either pmac75 or pmac9 exhibited a distinctive polar fluorescence (Fig. 3). This suggests that localisation might be directed by the EspC secretion machinery leading to a fusion protein exposed at the bacterial surface or accumulating in the periplasmic space due to a disturbance in part of EspC.

Autotransporter accessory factor mutant cells display multiple and polar localisation of EspC AT at the bacterial surface under green light illumination— To examine the effect of the accessory factors identified in a previous study to play a role in EspC secretion, mutants in their encoding genes (Δ*sapA,* Δ*nei,* Δ*proW,* Δ*ybgC,* Δ*ygjE,* Δ*abrB and* Δ*slyB*) were transformed with pmac9, and samples were prepared to observe under fluorescent microscope. In addition, the control (MG1655+pmac9) was set up to compare and determine if there are any significant difference in pmac fluorescence and behaviour. Under green light, the fluorescent microscope reveals that

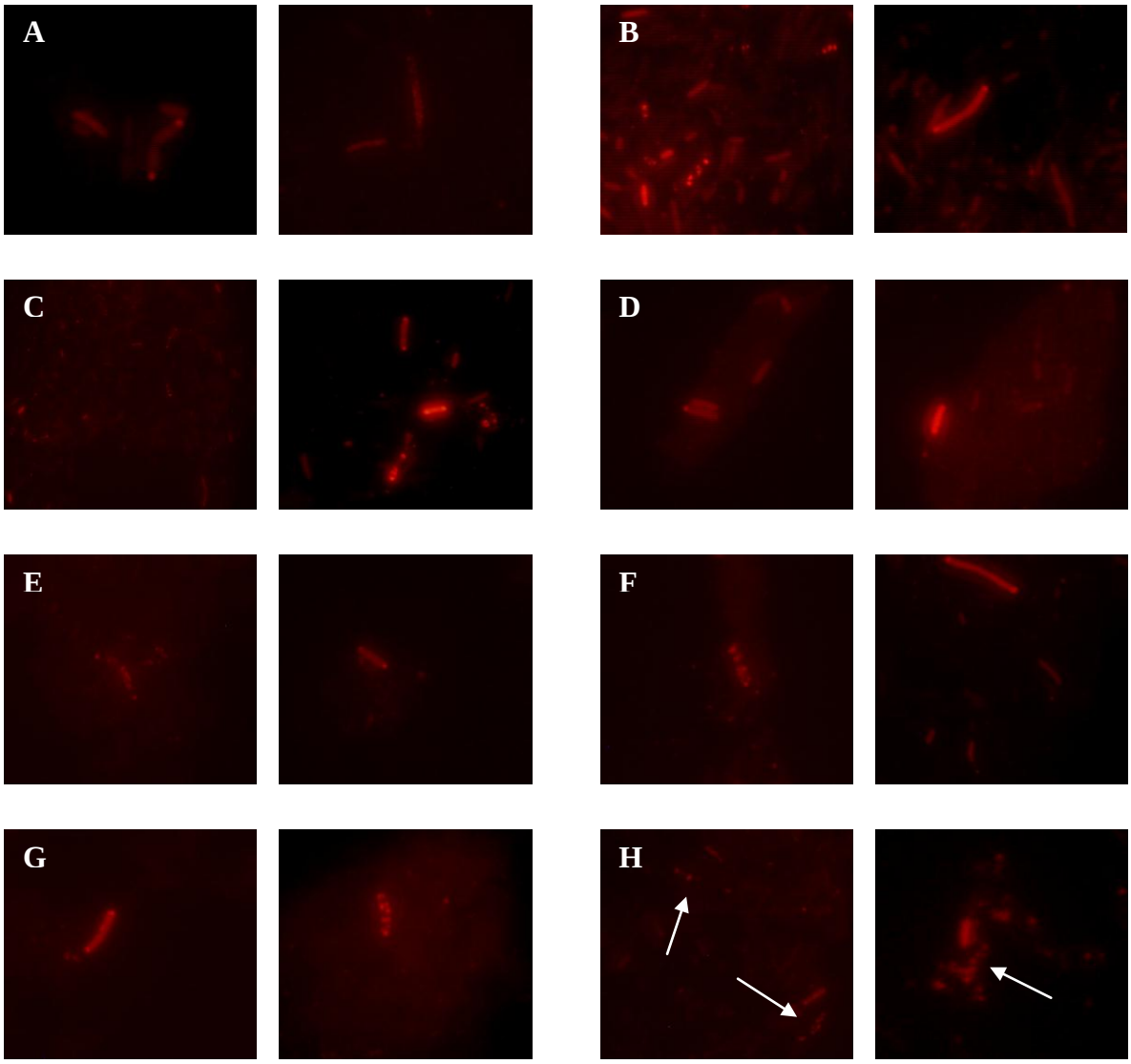
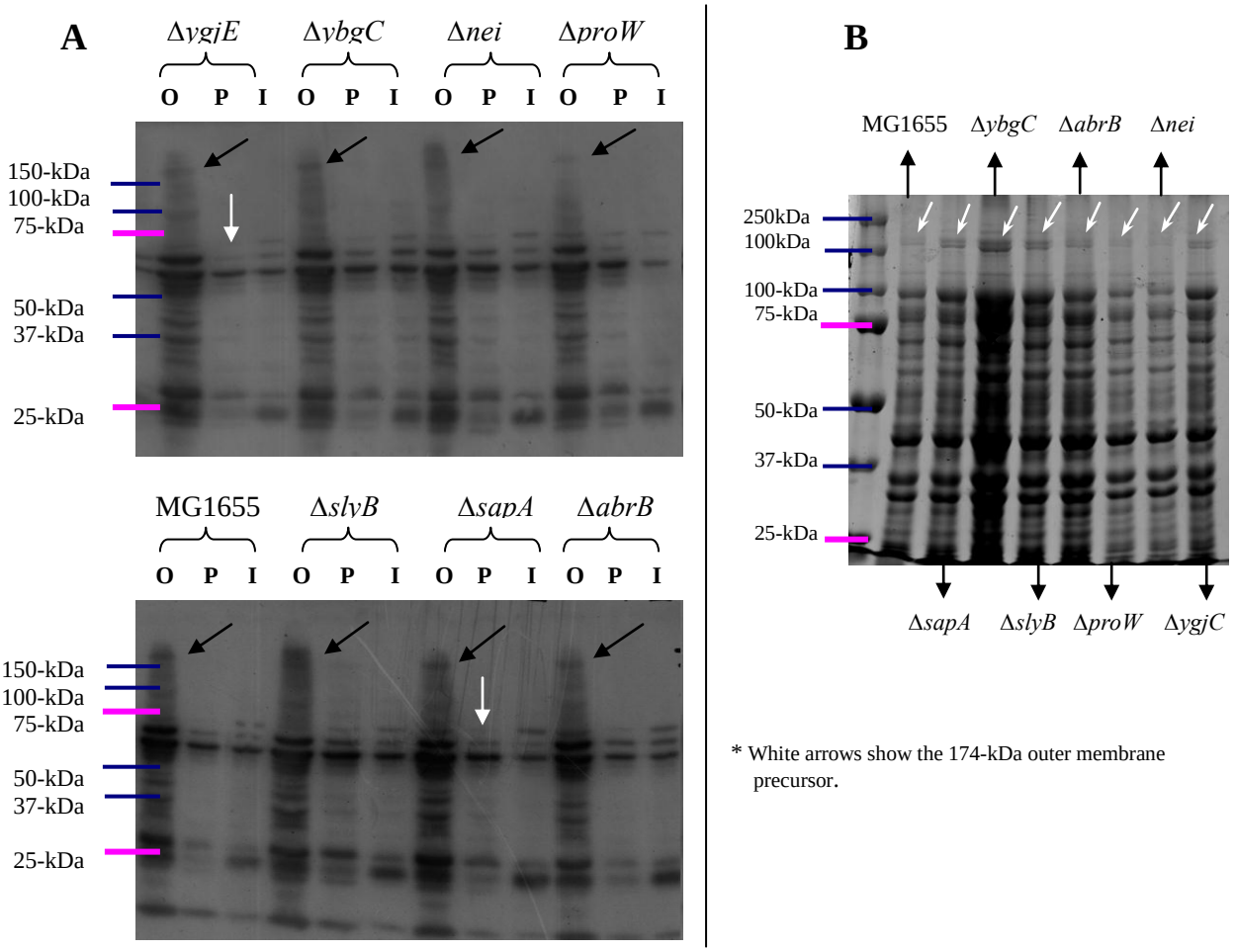


FIG. 4: Fluorescent microscope images indicate localisation of multiple EspC secretions in the mutant cells and detection of polar secretions in the all mutants. All mutants contain pmac9 construct and the cells were illuminated under green light and induced with arabinose. **A**, MG1655 Δ*abrB*. **B**, MG1655 Δ*nei*. **C**, MG1655 Δ*proW*. D, MG1655 Δ*sapA*. **E**, MG1655 Δ*slyB*. **F**, MG1655 Δ*ybgC* and **G**, MG1655 Δ*ygjE*. **H,** the control MG1655 *pmac9*, presence of multiple secretions at the bacterial surface (white arrows).

all the mutants expressed a kind of multiple localisation of the EspC AT (Fig. 4), possibly suggestive of inclusion body formation. Interestingly, the control also displayed this feature (Fig. 4H). There also appeared to be a significant reduction in the number of polar located EspC-mcherry in the all mutants compared to the control (Fig. 3).

Western blot and coomassie stain reveal detection of EspC AT at the bacterial outer membrane— Since microscope images illustrated that localisation of EspC-mcherry AT at the bacterial pole or at multiple locations, it is possible that this occurs in the outer membrane. Western blotting of whole cell fractions (cytoplasm, periplasm, inner membrane and outer membrane) of each mutant and the control was preformed in order to track the secretion steps and to confirm such localisation. All secretion accessory mutants and the control cells were fractionated using osmotic shock, spheroplast and Cambridge protocols as needed in order to separate the cell compartents, and then analysed by western blot. As expected, a whole length of EspC-mcherry precursor 174-kDa (Fig. 1) was observed in the outer membrane fraction in both mutants and the control (Fig. 5A), suggesting that localisation was externally presented. To confirm these results and



* White arrows show the 174-kDa outer membrane precursor.

FIG. 5: Western blotting and coomassie stain analysis reveal outer membrane localisation of the whole pmac precursor. *E coli* strain MG1655 and its derived AT accessory mutants (Δ*nei*, Δ*proW*, Δ*sapA*, Δ*ybgC*, Δ*slyB*, Δ*abrB* and Δ*ygjE*) containing pmac9 were grown to OD_{600} = 1.2 and fractionated into outer membrane (O), inner membrane (I) and periplasm (P). Each fraction was applied to a 7% SDS PAGE and western blot with anti-dsRed as primary antibody and anti-rabbit IgG peroxidase as secondary antibody. **Panel A**, shows all the strains and fractions as indicated. **Panel B**, compares outer membrane frations on SDS PAGE gel. The position of the whole EspC-mcherry precursor (174-kDa) is indicating by black arrows in panel A and by white arrows in panel B. the white arrows in panel A show the precursor of the degradaded 70.5-kDa portion. The relative position of molecular weight size markers are indicating in kDa alongside each gel.

to be more precise, two experiments were set up (i) just an outer membrane extracts were applied to a SDS PAGE gel. Similarly, 174-kDa EspC-mcherry bands were detected in all samples (Fig. 5B), trypsin treatment was preformed to whole cell extract of both DH5α containing mcherry protein and MG1655 containing pmac9 construct to see if there are any external protein being digested or no by the enzyme. As expected, the enzyme did not digest the mcherry protein in the first sample, whereas the outer membrane proteins in the second sample were completely digested compared to the control (Fig. 6), suggesting that localisation of mcherry it self was inside the cell, while localisation of the pmac9 protein whether the whole precursor or degraded portions was definitely external localised. In addition, a 70.5kDa precursor (Fig. 1) was observed strongly in some mutant fractions such as in the inner membrane of Δ*sapA*, Δ*nei* and Δ*ybgC*, whereas it seems to be almost absent in some others such as in the periplasm of Δ*ygjE* and Δ*sapA* (Fig. 5A). Thus it can be concluded that some mutants may show protein localisation defects. Interestingly, this precursor was also located in the outer membrane extract of all strains including the control, suggesting that it might represent protein aggregations since its size predicts that it lacks the β-barrel domain which in turn predicts that it should therefore not be found in this fraction. The presence of aggregated protein in the outer membrane fractions, despite efficient separation of the cellular compartments is likely to result from the nature of the

fractionation procedure which relies on detergent solubilisation and centrifugation. When protein aggregates are present, this is an unsuitable fractionation technique since such proteins have altered density and solubility.

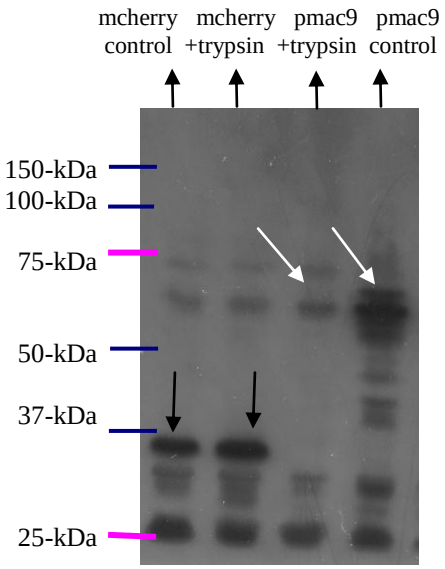


FIG. 6: Trypsin digestion. *E coli* strains DH5α containing mcherry fluorescent protein and MG1655 containing pmac9 were grown to OD_{600} = 6 and then pellet resupended in 600 µl of 100mM Tris-HCL pH 8.0 followed by adding 50µl of trypsin to 500µl and the rest 100µl left as control. After 1hr incubation at 37˚C, pellet resuspended in 1X SDS PAGE buffer for western analysis. Black arrows indicate the 34-kDa mcherry bands non digested. White arrows show digestion of the outer membrane proteins by the trypsin comparing to the control.

DISCUSSION

The red fluorescent protein, mcherry, was successfully inserted into the passenger domain of the EspC AT, and the localisation of the fusion protein was monitored by fluorescent microscopy as well as a combination of cellular fractionation and Western blotting. These analyses revealed surface exposure of mcherry directed by signals within EspC, plus a range distribution patterns dependent upon the host cell.

The efficiency of cloning the DNA encoding fluorescent proteins was improved by using antarctic phosphatise to treat the digested pladc1 vector. It works by removing the 5` phosphate group on both vector termini to (i) prevent the vector from self religating (since it lacks the 5` phosphate group, the 3`-OH group would be unable to form a phosphodiester bond). Therefore, the possibility of vector relegation becomes very low (ii) give opportunity for the digested PCR product (*egfp* & *mcherry*) to ligate into the plasmid resulting in successful cloning. This improved strategy increased the number of transformants isolated from 4 to 14.

Parallel attempts to tag GFP into EspC were probably unsuccessful because GFP has to be correctly folded to fluoresce. As EspC directs secretion via the Sec pathway, improper folding in the periplasm may have occurred preventing fluoresence. This can be avoided by translocating GFP in a fully folded conformation via the twin-arginine translocase (Tat) pathway as illustrated by its active fluorescent state when fused to the twine-arginine signal peptide of the trimethylamine N-oxide reductase (TorA). Proteins exported by the recently discovered Tat pathway contain special signal peptides with an essential twin-arginine motif that distinguishes them from Sec-dependent signal peptides (39). The finding that fused GFP export was almost abolished in Tat deletion mutants supports this (40). However, a very recent study illustrated that GFP was active and fluorescent when tagged to organophosphorus hydrolase (OPH) as a passenger domain for the *E coli* AT called adhesion-involved-in-diffuse-adherence (AIDA-I)(41). Since this AT utilizes Sec-dependent secretion, AIDA-I must differ from EspC in some way that confers it with an improved capability to translocate large passenger domains. As such, AIDA-I has been used as a monitoring tool to present heterologous macromolecule passenger proteins on the bacterial surface (41).

ATs from a wide variety of Gram-negative bacteria, for instance IcsA and SepA of *Shigella flexneri*, AIDA-I of diffusely adherent *E coli* and BrkA of *Bordetella pertussis*, are localised at the bacterial pole. This suggests that a specific polar secretion system exists (27). It has been proposed that such localisation is dependent on the presence of a complete lipopolysaccaride (LPS) on the bacterial membrane, despite some ATs such as BrkA being polar even in absence of LPS (27). These data support the results obtained in this study which pointed out that EspC AT was localised at the pole of bacterial surface in both wild type and AT

accessory factor mutant *E. coli* strains. In this study, it has been confirmed that localisation was at the bacterial surface rather then in the periplasm using both western blot analysis which detects the full length of the EspC precursor in the outer membrane fraction, and extracellular trypsin digestion which showed that these outer membrane proteins were completely digested.

Although using trypsin to digest proteins localised at the outer membrane produced encouraging results, the outer membrane protein recognized by anti-dsRed that was completely digested by trypsin (Fig. 6) would be predicted from its size (70.5kDa) to lack the β-barrel domain. This means that it should not be found in the outer membrane, and it is currently unclear why it is found here. The formation of inclusion bodies containing this 70.5kDa EspC-mcherry protein might explain why it co-fractionates with the outer membrane using the techniques employed in this study because these large aggregates would be insoluble in the detergents and pellet under the centrifugation conditions used. This could be avoided by using floatation sucrose gradients which provide more reliable and clear separation of outer membrane, inner membrane as well as membrane-associated proteins which float up the gradient, whilst large and soluble protein aggregates stay at the bottom (38). However, aggregate formation would not explain why this protein is exposed on the cell surface. Further clues to the localisation of the 70.5kDa protein may be gleaned from N-terminally sequencing it.

Although localisation of EspC-mcherry in distinct foci in *E coli* using fluorescent microscopy could be indicative of inclusion body information, indirect immunofluorescence of the NaIP AT of *Neisseria meningitidis* showed a similar pattern of multiple localisation on the surface of *Neisseria meningitidis.* The scattering observed over the cell surface without any particular pattern of distribution, suggested that localisation of neisserial ATs occurred at multiple distinct sites around the surface of coccoid bacteria. This might derive from the complex history of the cell envelope and its relationship to the cell division site. In contrast the localisation of this AT was more polar when produced in *E coli* (27), leading to the suggestion that polar localisation of ATs in general might occur by alternate pathways resulting in secretion at places away from the pole (27). Moreover, in spherical *E coli* that lack MreB, which is the bacterial cytoskeletal protein which plays an important role in the formation of the helical structures along the long axis of the bacterial cells, IcsA AT was localised at multiple sites (26, 42). This suggests that MreB restricts certain polar material to defined sites within the cells, so, in the absence of MreB, formation of ectopic or multiple sites take place around the cell containing polar material which could represent a defect in the aggregation of polar material at polar sites (42).

To conclude, mcherry protein has successfully inserted into EspC AT. This fusion strategy would introduce a potential tool for future studies to monitor and track protein secretion. All mutant strains and the wild type containing the construct appear to have polar and multiple site localisation of EspC-mcherry protein under fluorescent microscopy. Western blot and SDS PAGE experiments carried out and confirmed that protein localisation is externally presented at the outer membrane. However, the presence of 70.0-kDa precursor, which is predicted to be lacking the β-barrel domain, on the outer membrane is unexpected. It is unknown yet whether this precursor represents a protein aggregate and thus further investigation using more sophisticated cellular fractionation techniques is required.

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