

Translocating under the radar: an investigation into the secretion pathway of EspC, a ‘classical’ autotransporter from enteropathogenic *E. coli*

Thesis submitted to the University of Nottingham for the degree of
Doctor of Philosophy

Marina Serdar, BSc.



January 2023

Declaration

I declare that, unless otherwise stated, the work presented in this thesis is my own. This work hasn't been submitted in whole (or part) for another degree at the University of Nottingham or any other learning institute.

- Marina Serdar, January 2023

COVID-19 Impact Statement

Due to this project's heavy reliance on imaging equipment and molecular cloning, coupled with the work towards this research mainly taking place during the COVID-19 pandemic, not all planned aspects of the project could be realised in time for submission. This was due to several months of lab closure and ongoing delays in reagent and equipment delivery (e.g. the production and delivery of the anti-MreB antibody, many SDM/cloning primers and material transfers from the Schultz lab).

Abstract

Autotransporter proteins (ATs) are a family of diverse, surface-accessible virulence factors produced by numerous Gram-negative bacteria and utilise the Type V Secretion System (T5SS). Conserved domains direct AT secretion, and it has recently been revealed that chaperones assist ATs across the cell membrane, disputing the 'auto' in their name. Elucidating the steps of AT secretion will help the development of new antivirulence therapies. This study follows previous findings that the cytotoxic EPEC AT EspC localises into helical patches along the bacterial cell during secretion. The helical pattern resembles those of the actin homologue MreB and the Sec translocon. Disruption of either resulted in an altered EspC localisation. Here, an extended range of Sec temperature-sensitive (ts) mutants was used to probe EspC localisation at different steps of IM translocation. SecA_{ts} and SecY_{ts} mutants exhibited bipolar localisation of EspC, whilst SecD_{ts} and SecF_{ts} yielded no detectable EspC, supporting the role of the Sec translocon in EspC targeting. Next, the colocalisation of EspC-mCherry and MreB-TC in *E. coli* MG1655 was detected using super-resolution Structured Illumination Microscopy. The two proteins remained associated following treatment with a MreB polymerisation inhibitor, A22. To map EspC interactions on its way out of the cell, steps were taken to photo-crosslink EspC with the pBpa unnatural amino acid to substantiate the microscopy findings and discover more protein partners. An extracellularly labelled EspC has also been constructed using SpyCatcher technology for studying EspC during EPEC infections of intestinal epithelial cells with the intention of illuminating its internalisation into host cells. By discerning the protein-protein interactions and elucidating the secretion pathway of EspC, the ultimate aim is to gain insight into ATs of related enteric pathogens to underpin the development of new antimicrobials. Finally, EspC secretion was tracked during treatment with a promising T3SS antivirulent (Aurodox). It was confirmed that its potency extends to EspC (T5SS) because it shares T3SS's transcriptional regulator (Ler).

Acknowledgements

Predaja ovog doktorskog rada nije samo moj uspjeh već uspjeh cijele moje obitelji, i svih učitelja i odgajatelja koji su me kroz godine bodrili, ohrabivali, tješili i nesebično pružali sredstva koja mi trebaju da se ostvarim. Nisam očekivala da ću biti ovoliko daleko od doma i prijatelja, ali ustrajem znajući da će današnje žrtve ishoditi bolju budućnost. Zahvalna sam svojim roditeljima, Vladi i Anamariji Serdar na ljubavi i požrtvovnosti kojima su mene i brata oblikovali u uspješne ljude. Hvala bratu Augustinu koji mi služi kao intelektualno nadahnuće i moralna podrška u trenucima kad poželim odustati. Hvala ostatku obitelji Serdar, poglavito Lini, Rafaelu, baki Ani, Vandi i Manfredu na ljubavi, smijehu i pomoći kad god ju trebam (i kad ju ne trebam). Hvala mojim zauvijek-prijateljima Uni Nešković, Dori Vrkić, Nini Orlović i Lovri Mirthu koji me poznaju bolje no što poznajem samu sebe. Bez vas ne bih imala hrabrosti boriti se za ono što želim.

I want to extend my thanks to Prof. Kim Hardie, who took a chance on this wide-eyed biomedical scientist more than four years ago. Without the guidance and encouragement she and Dr Christopher Penfold provided throughout the years, I would've given up. Thank you to the wonderful team making up the C-floor lab in BDI – each and every single one of you taught me something new (apologies for all the stupid questions, though). Thank you, Dr Jeni Lockett, for not giving up after I'd say: "I'm fine." – it meant a lot to be seen. To my collaborator, Dr Robert Markus, thank you for teaching me so much about microscopy and helping me discover a new love for fluorescence imaging. I'd also like to thank Dr Jack Leo, Dr Rebecca McHugh and the Schultz lab for providing me with the necessary resources to bring new techniques into our lab. To my dearest Nottingham friends – Claire Laxton, Isobel Blower, Dr Maria Papangeli, Franka Zlatić, Dr Dean Walsh, Dr Samuel Fenn and Prof. Victoria Chapman - thank you for your understanding, unquestioning support, enthusiasm and all the good times. Finally, a special 'thank you' goes to my soulmate William E. M. Richardson, for being the source of all my strength.

List of Contents

Abstract	3
Acknowledgements	4
List of Contents	5
List of Figures	9
List of Tables	13
Abbreviations	14
1. Introduction	19
1.1. <i>Escherichia coli</i> – friend and foe	19
1.1.1. Enteric pathovars of <i>E. coli</i>	19
1.2. Enteropathogenic <i>E. coli</i> – EPEC.....	20
1.3. Bacterial secretion systems in Gram-negative bacteria ...	21
1.3.1. Transport across the inner membrane (IM)	22
1.3.2. Transport across the outer membrane (OM)	23
1.4. Serine protease autotransporters of Enterobacteriaceae (SPATEs)	26
1.4.1. EspC.....	29
1.4.2. The signal peptide	30
1.4.3. Passenger.....	31
1.4.4. Translocator	33
1.5. The current T5aSS secretion model	34
1.6. BAM & TAM dilemma	46
1.7. Spatial regulation by MreB	47
1.7.1. Subcellular localisation of proteins	48
1.8. Techniques for the study of protein interactions	49
1.8.1. Super-resolution microscopy	49

1.8.2.	Protein labelling techniques for <i>in vivo</i> imaging	50
1.8.3.	Extracellular labelling using SpyCatcher technology	51
1.8.4.	<i>In vivo</i> photo-crosslinking using unnatural amino acids	52
1.8.5.	Antivirulents	53
1.9.	Hypothesis and Aims	54
2.	Methods	55
2.1.	Bacterial strains	55
2.2.	Plasmids	56
2.3.	Bacterial culture and antibiotics	57
2.4.	Growth conditions	58
2.4.1.	Preparation of temperature-sensitive SecYEG mutants	58
2.5.	DNA techniques	59
2.5.1.	Plasmid DNA extraction and purification	59
2.5.2.	DNA electrophoresis	59
2.5.3.	Site-directed mutagenesis (SDM) polymerase chain reaction (PCR)	59
2.5.4.	Kinase, ligase & <i>DpnI</i> (KLD) treatment	61
2.5.5.	T4 Polynucleotide Kinase (PNK) and ligase treatment	63
2.5.6.	DNA assembly	64
2.5.7.	Preparation of electrocompetent <i>E. coli</i> cells	65
2.5.8.	Electroporation	65
2.5.9.	Chemical transformation	66
2.6.	Protein techniques	66
2.6.1.	Whole-cell protein preparation	66
2.6.2.	SDS-PAGE electrophoresis	67
2.6.3.	Western Blot	68

2.6.4. Nickel Immobilised Metal Affinity Chromatography (Ni-IMAC) protein purification of SpyCatcher proteins	69
2.7. SpyTag/SpyCatcher experiments	71
2.8. Quantification of EspC at the OM	71
2.9. Detection of EspC in supernatants following incubation with Aurodox	72
2.10. Reverse transcription-quantitative PCR (RT-qPCR)	72
2.10.1. RNA extraction	73
2.10.2. Reverse transcription for the synthesis of cDNA	73
2.10.3. RT-qPCR and result validation	73
2.11. <i>In vivo</i> photo-crosslinking using pBpa unnatural amino acid	74
2.12. FIAsh-EDT ₂ labelling of tetracysteine-tagged recombinant proteins	77
2.13. MreB disruption with MreB polymerisation inhibitor A22	77
2.14. Fluorescence microscopy	78
2.15. Super-resolution Structured Illumination Microscopy ..	78
2.15.1. Mounting methods	78
2.15.2. Cell fixation	79
2.16. Figure preparation	79
3. Validation of EspC-mCherry and EspC-TC constructs to study protein localisation in <i>E. coli</i> MG1655	80
3.1. Introduction	80
3.2. Results	84
3.2.1. Purification of SpyCatcher-sfGFP fusions	85
3.2.2. Introducing SpyTag in the middle of the EspC passenger	88

3.2.3. Placing the ST tag closer to the N-terminus of the EspC passenger	91
3.2.4. An attempt to introduce the SpyTag into EspC using DNA assembly	96
3.2.5. The introduction of the TC tag into mCherry	98
3.3. Discussion	100
4. EspC's helical localisation depends on the intact cytoskeleton and the Sec machinery	103
4.1. Introduction	103
4.2. Results	107
4.2.1. A range of Sec temperature-sensitive mutants disturb EspC localisation	108
4.2.2. SR-SIM confirmed that EspC and MreB colocalise... ..	111
4.2.3. Optimisation of SR-SIM image acquisition for 3D imaging.....	115
4.2.4. EspC-mCherry remains partially colocalised with MreB-TC when MreB polymerisation is inhibited by A22	118
4.2.5. <i>In vivo</i> photo-crosslinking of EspC.....	121
4.3. Discussion	127
5. Insights into the inhibition of EspC secretion using a T3SS inhibitor Aurodox	133
5.1. Introduction	133
5.2. Results	136
5.2.1. The T3SS inhibitor Aurodox inhibits EspC secretion .	136
5.2.2. EspC inhibition by Aurodox occurs at the transcriptional level.....	137
5.2. Discussion	142
6. Conclusions and future directions	144
7. References	149

List of Figures

Figure 1.1. Electron microscopy image of an enteropathogenic <i>Escherichia coli</i> adhered to intestinal epithelial cells in the small intestine, forming an A/E lesion..	21
Figure 1.2. Classification of bacterial secretion systems according to Christie (2019).	23
Figure 1.3. A detailed schematic of EspC, used as the prototypical member of the SPATE subfamily of classical ATs.	27
Figure 1.4. Evolutionary relationships between SPATEs according to their passengers and known target substrates.	29
Figure 1.5. A sequential schematic of ‘classical’ autotransporter biogenesis in the cytosol and its inner membrane translocation.	36
Figure 1.6. A sequential schematic of ‘classical’ autotransporter secretion steps in the periplasm.	41
Figure 1.7. A sequential schematic of ‘classical’ autotransporter outer membrane translocation.	43
Figure 1.8. A sequential schematic of ‘classical’ autotransporter passenger domain surface exposure.	45
Figure 1.9. An image can be expressed as a sum of sines and cosines of different amplitudes, known as the Fourier transform.	50
Figure 1.10. The principle of the SpyTag-SpyCatcher system.	52
Figure 3.1. SpyCatcher-sfGFP is available in active (E78) and inactive (Q78) form.	81
Figure 3.2. SpyCatcher-sfGFP was successfully purified using Ni-IMAC.	86
Figure 3.3. SpyCathcerEQ-sfGFP was successfully purified using Ni-IMAC.	87
Figure 3.4. Cloning strategy for the incorporation of the SpyTag polypeptide into EspC and EspC-mCherry.	88
Figure 3.5. The PCR step of site-directed mutagenesis was successful SpyTag insertion confirmed in control EspC constructs.	89

Figure 3.6. The SpyTag sequence was not successfully inserted into either of the EspC-mCherry constructs.....	91
Figure 3.7. Cloning strategy for the insertion of SpyTag at the N-terminus of EspC and EspC-mCherry.	92
Figure 3.8. The predicted structure of EspC generated by AlphaFold.	93
Figure 3.9. SpyTag insertion between D59 and N60 yielded many transformants but was ultimately unsuccessful.	94
Figure 3.10. Sequence analysis of the SpyTagged transformants following site-directed mutagenesis revealed non-specific binding of the SDM primers.....	95
Figure 3.11. Strategy for the incorporation of SpyTag into EspC constructs using Hi-Fi DNA assembly	96
Figure 3.12. HiFi DNA Assembly was used to obtain EspC and EspC-mCherry mutants after unsuccessful SDM attempts.....	97
Figure 3.13. The SpyTag wasn't successfully amplified following HiFi DNA assembly.....	98
Figure 3.14. A TC tag was successfully engineered into mCherry using site-directed mutagenesis.....	99
Figure 4.1. Resolution enhancement by Structured Illumination Microscopy (SR-SIM).	105
Figure 4.2. EspC-mCherry localisation is not disrupted at high temperatures (42° C) in <i>E. coli</i> MG1655, but its localisation is altered in SecA and SecY mutants at 42° C.....	109
Figure 4.3. EspC-mCherry localisation is not disrupted at low temperatures (25° C) in <i>E. coli</i> MG1655, but it is not detectable in SecD and SecF mutants at 25° C.	110
Figure 4.4. Initial indication that EspC interacts with MreB.	111
Figure 4.5. Live <i>E. coli</i> MG1655 simultaneously harbouring plasmids encoding EspC-mCherry and MreB-TC do not consistently exhibit the expected red and green fluorescence, suggesting the two fluorophores are detected as separate signals.....	112

Figure 4.6. 2-dimensional SIM confirmed that EspC-mCherry and MreB-TC colocalise in discrete patches around the <i>E. coli</i> MG1655 cell	113
Figure 4.7. 3-dimensional super-resolution Structured Illumination Microscopy reveals <i>E. coli</i> MG1655 cells producing EspC-mCherry (red) and MreB-TC (green) exhibit cell flattening.....	114
Figure 4.8. (A) Brownian motion can be minimized by applying the adhesive polylysine to improve live 3D SIM imaging of EspC-mCherry, but it reduces the effectiveness of the antibleaching agent CFM3. ...	116
Figure 4.8. (B) The use of Poly-D-Lysine as a coverslip adhesive successfully prevents Brownian motion in the samples when compared to the cells mounted with the photo-stable CFM3.....	117
Figure 4.9. EspC-mCherry colocalises with MreB-TC after inhibition of MreB actin polymerisation with A22 (2D SR-SIM)	118
Figure 4.10. 3D SR-SIM analysis of fixed <i>E. coli</i> MG1655 reveals EspC-mCherry (red) partially localises adjacent to MreB-TC (green) at the IM after the treatment with A22.....	119
Figure 4.11. There is no fluorescence bleedthrough from the green (488 nm) to the red (561 nm) channel caused by A22-treatment and cell fixation	120
Figure 4.12. Outline of the experimental procedure to photo-crosslink EspC to proteins it interacts with using pBpa..	124
Figure 4.13. Crosslinking of EspC via pBpa did not yield distinct protein complexes for further analysis.	126
Figure 5.1. An overview of the <i>ler</i> transcriptional regulatory cascade. Before pathogenesis is initiated, EPEC and EHEC virulence genes are silenced by H-NS which binds DNA at two sites upstream of the <i>ler</i> promoter.	134
Figure 5.2. EspC secretion decreases when treated with the T3SS inhibitor Aurodox.....	137
Figure 5.3. Standard amplification curves were created for <i>espC</i> and <i>rrnB</i> using EPEC genomic DNA.	138
Figure 5.4. Final concentrations of <i>espC</i> qPCR primers were determined according to their amplification of EPEC gDNA.	138

Figure 5.5. RNA degradation was observed for EPEC E2348 samples incubated with 5 µg/mL Aurodox	139
Figure 5.6. The results of relative quantifications of gene expression show a 1.67-fold decrease in <i>espC</i> transcription in samples treated with sub-bacteriostatic levels of the T3SS inhibitor Aurodox.....	141
Figure 5.7. An overall decrease in <i>espC</i> mRNA qPCR detection is observed in samples which received Aurodox treatment.....	141

List of Tables

Table 1.1. Members of Serine Proteases Autotransporters of Enterobacteriaceae don't share a single point of origin on the bacterial chromosome.....	28
Table 1.2. Periplasmic chaperone – autotransporter protein-protein interactions reported so far	40
Table 2.1. Bacterial strains used in this study.	55
Table 2.2. Plasmids used in this study	57
Table 2.3. Oligonucleotide primers used for PCR amplification and site-directed mutagenesis..	61
Table 2.4. Site-directed mutagenesis reaction components and thermocycling conditions used for insertions.	62
Table 2.5. KLD mixture recipe.....	62
Table 2.6. Post-PCR processing steps used to increase the transformation efficiency of mutants generated by SDM.	63
Table 2.7. Reaction components and thermocycling conditions used for colony PCR.	64
Table 2.8. Recipes used to prepare reagents for SDS-PAGE.....	67
Table 2.9. Recipes for reagents used in Western blotting.....	68
Table 2.10. Reagents used to purify SpyCatcher WT/EQ using Ni-IMAC.	69
Table 2.11. Ni-IMAC protein purification procedure using a benchtop peristaltic pump..	70
Table 2.12. Recipe for the minimal glycerol media used to express EspC with an amber codon (TAG) engineered at codon 951 of the <i>espC ori</i>	75
Table 2.13. Table outlining the constructs and growth conditions used to perform pBpa photo-crosslinking.	76
Table 3.1. EspC-STa (pLA33C1ST1) and EspC-STb (pLA18c1ST1) successfully bind SpyCatcher-sfGFP after OM translocation.....	90
Table 4.1. Table outlining the constructs and growth conditions used to perform pBpa photo-crosslinking	122
Table 5.1. Amplification conditions for RT-qPCR	140

Abbreviations

A – alanine

Aa – amino acid

A22 – S-83,4-dichlorobenzene-isothiourea

A/E – attaching and effacing [lesions]

aEPEC – atypical Enteropathogenic *E. coli*

AC – autochaperone

AHTC – anhydrotetracycline

AIEC – adherent invasive *E. coli*

Amp – ampicillin

APEC – avian pathogenic *E. coli*

APS – ammonium persulfate

AT – autotransporters

ATP – adenosine triphosphate

BAL – British anti-Lewisite (dimercaprol)

BAM – β -barrel assembly machinery

BB – β -barrel

BFP – bundle forming pilus

bp – base pairs [nucleotides]

C – cysteine

cDNA – complementary deoxyribonucleic acid

Cm – chloramphenicol

C_T – cycle threshold

CTD – C-terminal domain

CV – column volume

D – aspartic acid

DAEC – diffusely adherent *E. coli*

DMEM – Dulbecco's Modified Eagle Medium

DMSO – Dimethyl sulfoxide

DNA – deoxyribonucleic acid

EAEC – enteroaggregative *E. coli*

pEAF – EPEC adherence factor plasmid

EDTA – ethylenediaminetetraacetic acid

EF-Tu – elongation factor - Tu

EHEC – enterohaemorrhagic *E. coli*

EIEC – enteroinvasive *E. coli*

EPEC – enteropathogenic *E. coli*

EQ – glutamate-glutamine [amino acids]

ESBL – extended-spectrum betalactamase

ESPR – extended signal peptide regions

ETEC – enterotoxigenic *E. coli*

F – phenylalanine

FAK – focal adhesion kinase

FIAsH-EDT₂ – fluorescein arsenical hairpin binder; C₂₄H₁₈As₂O₅S₄

FP – fluorescent protein

G – glycine

GA – glutaraldehyde

gDNA – genomic deoxyribonucleic acid

GDP – guanosine-5'-diphosphate

GFP – green fluorescent protein

GTP – guanosine-5'-triphosphate

H – histidine

HeLa – Henrietta Lacks [cell line]

HEPES – 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethane-1-sulfonic acid

HIV – human immunodeficiency viruses

HAS – Health and Safety Authority

IECs – intestinal epithelial cells

IB – inclusion body

IPTG – isopropyl β-D-1-thiogalactopyranoside

K – lysine
KLD – kinase, ligase, DpnI [treatment]
L – leucine
LA – local adherence
LB – Luria Bertani
LEE – locus of enterocyte effacement
LPS – lipopolysaccharide
M – methionine
MBP – maltose-binding protein
MFP-ABC – membrane fusion protein – ATP-binding cassette
min - minute
NEB – New England Biolabs
NF – nuclease-free
Ni-IMAC – nickel immobilised metal affinity chromatography
Ni-NTA – nitrilotriacetic nickel²⁺ ion
OD – optical density
OM – outer membrane
OMP – outer membrane protein
ORF – open reading frame
P – proline
PAGE – polyacrylamide gel electrophoresis
pBpa – p-benzoylphenylalanine
PAH – polycyclic aromatic hydrocarbons
PAI – pathogenicity island
PBS – phosphate-buffered saline
PCR – polymerase chain reaction
PG – peptidoglycan
PL – pertactin-like [region]
PNK – polynucleotide kinase
POTRA – polypeptide-transport-associated [domains]

PPI – protein-protein interactions
PVDF – polyvinylidene fluoride
Q – glutamine
qPCR - quantitative polymerase chain reaction
RIF – regions of increased fluidity
RIN – RNA integrity number
RNA – ribonucleic acid
rRNA – ribosomal ribonucleic acid
ROI – region of interest
RPM – rotations per minute
RS – reverse synthetase
RT – reverse transcription
RT-qPCR – reverse transcription quantitative polymerase chain reaction
S – serine
SDM – site-directed mutagenesis
SDS – sodium dodecyl sulfate
sfGFP – super-folder green fluorescent protein
SIM – structured illumination microscopy
Sm - spectinomycin
SLAT – SPATE-like autotransporters
SP – signal peptide
SPATE – serine protease autotransporters of Enterobacteriaceae
SR – super-resolution
SRP – signal recognition particle
SS – secretion system
ST – SpyTag
STEC – Shiga toxin-producing *E. coli*
T1SS – type I secretion system
T2SS – type II secretion system
T3SS – type III secretion system

T4SS – type IV secretion system
T5SS – type V secretion system
T6SS – type VI secretion system
T7SS – type VII secretion system
T8SS – type VIII secretion system
T9SS – type IX secretion system
T_a – [primer] annealing temperature
T_m – melting temperature
TAA – trimeric autotransporter adhesins
TAE – Tris base, acetic acid, EDTA
TAG – amber stop codon
TAM – translocation and assembly module
Tat – twin-arginine translocation
TBS – Tris-buffered saline
TBST – Tris-buffered saline, Tween®20
TC – tetracysteine
Tc – tetracycline
TCA – trichloroacetic acid
TEMED – N,N,N',N'-tetramethylethane-1,2-diamine
tEPEC – typical enteropathogenic *E. coli*
Tris – tris(hydroxymethyl)aminomethane
tRNA – transfer ribonucleic acid
TS – transport systems
UAA – unnatural amino acid
UV – ultraviolet [light]
v/v – volume per volume
w/v – weight per volume
WHO – World Health Organization
WT – wild-type

1. Introduction

1.1. *Escherichia coli* – friend and foe

E. coli is one of the most frequently occurring commensals in our intestines – it helps break down & absorb food. For decades, it has been used to manipulate genomes and express recombinant DNA. Unfortunately, its ease of plasmid exchange and propensity for mutation also make it an adaptable and effective pathogen. Communicable diseases, including bacterial infections, are prominent among the top 10 global causes of mortality, with the potential to become more prominent in the next few years (1). Antibiotic resistance is rising due to globalisation and disparate national practices, causing a substantial clinical and economic burden on healthcare systems worldwide due to the increasing inability to cure ‘common’ bacterial infections (2). More reports are emerging on the increase of *E. coli* bacteraemia and the spread of extended-spectrum β -lactamases (ESBL)-producing *E. coli*, making the search for new antimicrobial targets to help treat multi-drug resistant Gram-negative bacteria a priority (3).

1.1.1. Enteric pathovars of *E. coli*

Child mortality caused by diarrhoeal disease remains a global burden, accounting for around 525 000 deaths of children under five years of age annually (4). Among deaths caused by communicable diseases, diarrhoeal disease is second only to respiratory infections. Diarrhoea leads to malnutrition, electrolyte deficiency and dehydration which, if left untreated, can have life-threatening outcomes, with small children and those immunocompromised or living with HIV being the most vulnerable. The most common bacterial aetiological agents of diarrhoeal disease in low-income countries are diarrheagenic *E. coli*, *Campylobacter jejuni*, *Salmonella enterica* and *Vibrio cholerae* (5). Diarrheagenic *E. coli* are typed according to how they adhere to or invade intestinal epithelial cells (IECs): enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli*

(STEC), enterohaemorrhagic *E. coli* (EHEC), Shigella/enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), enterotoxigenic *E. coli* (ETEC) and adherent invasive *E. coli* (AIEC). The prevalence of specific pathovars varies with geographical location, with EPEC dominating Chile, Venezuela, Uruguay and Argentina (6). Another study in sub-Saharan Africa and East Asia identified EPEC as an aetiological agent responsible for moderate-to-severe diarrhoeal disease with higher risks of case death in infants (7).

1.2. Enteropathogenic *E. coli* – EPEC

EPEC are distinct by their ability to form attaching and effacing (A/E) lesions on the intestinal epithelial cells (IECs) using virulence factors trafficked by bacterial secretion systems into host epithelia (8). These lesions result in the loss of microvilli, cytoskeletal changes and cell dysmorphia induced by actin polymerisation, forming a cup-like pedestal underneath the bacterium (Fig. 1.1.). Genes required for lesion development are located on a pathogenicity island (PAI) called the locus of enterocyte effacement or LEE (9). The lesions result from the interaction between intimin and its receptor Tir, both encoded in LEE. When secreted, Tir utilises the Type III Secretion System (T3SS) to attach to IECs and act as a receptor for intimin. Their interactions trigger the host cell's Src-family kinase c-Fyn to phosphorylate Tir, leading to actin accumulation and microvilli effacement (10, 11). EPEC is subclassified into typical (tEPEC) and atypical (aEPEC) based on whether or not they contain the *E. coli* adherence factor plasmid (pEAF), which encodes the bundle forming pilus (BFP) required for microcolony formation (12). The presence of pEAF is thought to be responsible for the localised adherence (LA) pattern displayed by tEPEC (13). Lacher *et al.* (2006) have postulated that aEPEC has likely evolved from tEPEC due to selective pressure to lose the EAF plasmid when carried by adult hosts, who are less likely to suffer from EPEC-caused gastrointestinal disease (13). Despite the loss of this LA plasmid, aEPEC strains became

more prevalent globally, suggesting that pEAF presence is not vital to EPEC virulence, but that its loss possibly aids immune evasion (14). The shift has been noted in developed and developing countries (15, 16). With the increase in the use of molecular biology techniques for diagnostic purposes, EPEC is currently estimated to be the cause of 5-10% of all 'paediatric diarrhoeal episodes' in the developing world (17).

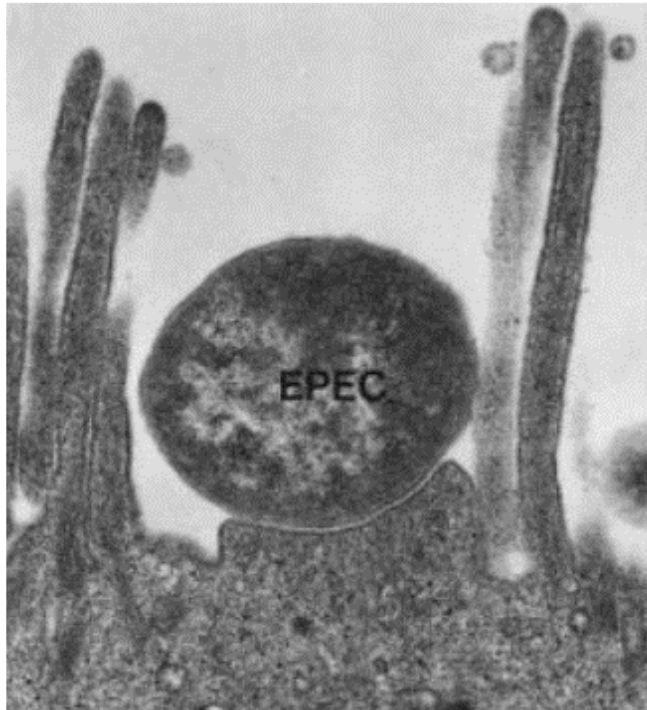


Figure 1.1. Electron microscopy image of an enteropathogenic *Escherichia coli* adhered to intestinal epithelial cells in the small intestine, forming an A/E lesion. It is surrounded by healthy microvilli. The figure was taken from Knutton *et al.*, 1987 (8).

1.3. Bacterial secretion systems in Gram-negative bacteria

Efficient protein transport is essential for bacterial survival as bacteria produce proteins for various purposes, including nutrient acquisition, structural integrity maintenance, division and host invasion in pathogenic strains. As a result, bacteria have evolved an array of transport systems which differ in complexity according to the proteins that utilise them and their final destination (18).

1.3.1. Transport across the inner membrane (IM)

1.3.1.1. Sec (general secretion) pathway

The Sec translocation system comprises cytosolic and membrane proteins which work together for successful translocation. It is used by most proteins to pass unfolded through the IM. There are two ways pre-proteins are delivered (targeted) to the Sec translocon. In co-translational IM targeting, reserved almost exclusively for IM proteins, the SRP (signal recognition particle) recognises hydrophobic regions of an N-terminal signal peptide as it exits the ribosome and transfers the nascent polypeptide chains to the Sec translocon. An investigation into the *E. coli* proteome revealed that co-translational IM targeting is only used for around 2% of secreted proteins, with the rest comprising only IM proteins, (19, 20). In post-translational targeting, a cytoplasmic chaperone, SecB, recognises a ~25 amino acid sequence (signal peptide/SP) at the N-terminus of a pre-protein, present in substrates destined for the periplasm (where they can stay or utilise another secretion system for subsequent translocation). SecB delays premature folding (together with another chaperone, DnaK) and directs the pre-protein to the SecA-SecYEG holoenzyme complex, where SecA recognises it (21, 22, 23). The binding of the pre-protein to SecA triggers the opening of the SecYEG channel, after which SecA provides energy to 'push' the pre-protein across the IM (see section 1.6.) (21, 24, 25). When most of the pre-protein passes through the SecYEG channel, the embedded SP is cleaved, releasing the rest of the pre-protein to the periplasm. Apart from the central Sec machinery and steps described here, other non-Sec proteins act to help insert pre-proteins into the membrane, e.g. the membrane insertase YidC (26, 27).

1.3.1.2. Tat pathway

Some pre-proteins require cytosolic cofactors for their post-translational modifications, so they fold before IM translocation. They utilise a separate translocation machinery, the twin-arginine translocation (Tat) system. Its secretion pathway consists of TatABC in *E. coli*, where TatBC binds the cargo proteins containing twin arginines present in their signal sequence and recruits TatA, which forms the channel in the IM to facilitate translocation (18, 28). Similar to Sec-secreted proteins, the translocated proteins can stay in the periplasm or use the Type II Secretion System (T2SS) system to cross the outer membrane.

1.3.2. Transport across the outer membrane (OM)

Since not all proteins are destined for the periplasm, Gram-negative bacteria have developed different secretion systems that span two to three membranes. These systems are still being investigated, with nine currently described and various subtypes emerging. Here, they are classified according to Christie into class I) two-step Gram-negative OM-spanning, II) two-step Gram-negative cell-envelope-spanning, III) one-step Gram-negative cell-envelope-spanning and IV) one-step Gram-positive cytoplasmic-membrane-spanning (Fig. 1.2.) (24).

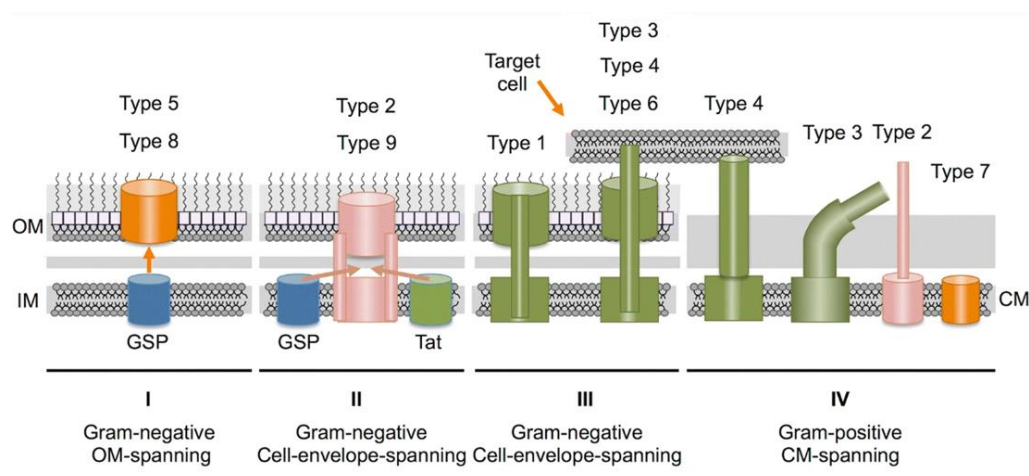


Figure 1.2. Classification of bacterial secretion systems according to Christie (2019). The Type VII Secretion System (T7SS) is a Class IV secretion system unique to Gram-positive bacteria and won't be discussed in this study (24).

The Type V Secretion System (T5SS) and Type VIII Secretion System (T8SS) comprise Class I and usually transport surface-associated proteins (24). The T5SS encompasses autotransporter proteins (ATs), widely conserved and structurally similar virulence factors which can vary drastically in their function. Interestingly, the T5SS subtypes emerged from structural variances and domain organisation instead of protein function (29). T5SS substrates comprise an N-terminal signal peptide, a central passenger and a C-terminal translocator. The 'classical' T5aSS is the most studied subtype and is discussed in detail in 1.4. Until recently it was accepted that after completing Sec-assisted IM translocation, the characteristic β -barrel domain encoded in the protein functions as the OM anchor through which the functional domain (passenger) is pulled through. Where the energy required for this step comes from was speculative. The secreted proteins can either remain tethered to the cell's surface or undergo auto-proteolytical cleavage to be released. Closely related to 'classical' are inverse ATs (T5eSS), characterised by their inversed domains, with N-terminal β -barrels and C-terminal SPs (30). T5bSS, also known as two-partner secretion, requires a pair of proteins where one functions as a translocator (TpsB), and the other is the functional passenger (TpsA) (31). Trimeric autotransporter adhesins (TAA) are secreted by T5cSS, with steps resembling that of T5aSS but involving larger, trimeric proteins that resemble 'lollipops' (32). T5dSS autotransporters relate to both 5a and 5b systems. They are distinguished by a structural difference in their β -barrel (closely resembling that of the T5b TpsB) and the fact that it transports only passengers with lipase/esterase activity (29). Not much is known about the recently discovered T5fSS, which seems exclusive to *Helicobacter pylori* (29). The T8SS is homologous to curli fibre assembly in Bacteroidetes and Proteobacteria. Curli components CsgA and CsgB utilise Sec to cross the IM and CsgE-G to cross the OM. CsgG forms a constitutively open translocation channel in the OM which extends into the periplasm, resembling a chamber (Fig. 1.2.). CsgE acts as the channel gatekeeper, letting through unfolded curli fibre subunits which assemble and mature into curli fibres at the OM (33, 34).

Class II Gram-negative cell-envelope spanning systems include the T2 and T9SS. The T2SS is a macromolecular complex made up of i) the OM pore (secretin), ii) the IM platform connected to the secretin, iii) a cytoplasmic ATPase which is recruited to the IM platform, and iv) a pseudopilus screw which pushes the T2SS substrates using energy generated at the IM (35). The T9SS has only been recently described; the current model proposes a Sec-dependent IM translocation followed by SP cleavage, after which the substrates use their C-terminal domain (CTD) to bind T9SS machinery and get passed around until they cross the OM channel Sov (36). At the OM, T9SS components swap their CTD and attach them to the lipopolysaccharide in a sortase-like manner (24).

Secretion systems developed to transport substrates in one step are grouped into Class III and comprise Types I, III, IV and VI Secretion Systems (T1SS, T3SS, T4SS and T6SS, respectively). T1SS consists of the ABC transporter, which is localised at the IM and contains a nucleotide-binding and a transmembrane domain. The membrane fusion protein – ATP binding cassette (MFP-ABC) complex recognises T1 substrates by their C-terminal translocation signal and recruits TolC, which inserts into the OM and extends through the periplasm to form a channel (Fig. 1.2.) (24).

The T3SS injectosome is a three-membrane spanning complex that allows effector proteins to be ‘injected’ directly into host cells. The injectosome assembly comprises a basal body, a needle component and the translocon (Fig. 1.2.) (37). The basal body spans both membranes to form a socket-like structure which consists of several rings and a rod with an encased needle-like filament that extends through the OM into the extracellular milieu. The needle component is hollow and permits the secretion of unfolded effectors into host cells, and it is regulated by the tip of the complex (18).

Three functionally unique T4 systems can traffic proteins and DNA (or protein-DNA complexes), having evolved from conjugative systems (38). Similarly to the T3SS, its central machinery can span multiple

membranes to deliver substrates directly into target cells, with additional factors (e.g. relaxases) mediating DNA transfer (Fig. 1.2.) (39). Three ATPases at the IM complex provide energy to propel the substrate through a narrow central channel connecting the IM and the OM T4SS complexes (40). The machinery of T6SS shares similarities with bacteriophage contractile tails, except it is inverted to eject substrates. It consists of a membrane complex (spanning both membranes), a baseplate and a tail (Fig. 1.2.). The tail is made up of a spike tube responsible for substrate recruitment and a sheath which contracts like a spring to push the spike tube towards the host cell membrane (24, 41). For a deeper insight into the complex types of machinery in bacterial secretion systems, please consider the excellent review by Christie (24).

1.4. Serine protease autotransporters of Enterobacteriaceae (SPATEs)

Autotransporter proteins (ATs) are a large family of Gram-negative virulence factors thought to initially encode all necessary information required to be secreted across the membranes, utilising the T5SS. Their simplistic structure led researchers to believe they translocate autonomously, using their signal peptide to direct crossing of the IM, while the β -barrel inserted itself into the OM and used energy derived from sequential folding of the passenger to extrude itself, facilitated by forming a hairpin loop (42). Evidence to the contrary, describing various essential protein interactions, emerges every so often, contradicting AT's 'auto-transporting' abilities, but the misnomer remains in use today (43). Research on AT secretion is hard to navigate because of the diverse nature of passenger functions and the fact ATs are widely conserved (as virulence factors present in various species, e.g. ATs of *E. coli* and *N. gonorrhoea*), making it difficult to unite discoveries from different organisms into a single secretion model. AT literature research is also negatively impacted by ambiguous nomenclature (44). Here, the terminology agreed upon by Drobnak *et al.* (2014) will be used, which

distinguishes in terms of protein architecture, domain function and sequence organisation (44).

ATs are an addition to the arsenal of virulence factors pathogenic *E. coli* already have at their disposal: they help with surface adhesion (e.g. AIDA-I, EhaA, intimin), biofilm formation (e.g. Ag43, EtpA, Eib), or cause cytotoxicity (e.g. EspC, EspP). A study conducted in 2002 analysed 120 ATs from 20 bacterial genera and found that 29 out of the 33 phylogenetic branches bearing ATs originate from a single phylogenetic group. Based on this, ATs have evolved through speciation and duplication events, with horizontal gene transfers being only rare evolutionary events (45).

Serine proteases of Enterobacteriaceae (SPATEs) are a subfamily of classical ATs grouped because they i) share up to 99% β -barrel sequence similarity, ii) are proteases whose passengers contain the GDSSGS motif in which S is the catalytic serine, iii) are autocatalytically cleaved after OM translocation (as supposed to remaining surface-tethered), iv) possess an extended signal peptide (present only in 10% of autotransporters, see section 1.4.2., Fig. 1.3.) and v) are secreted in abundance by the parental strain (46). SPATEs can be encoded on

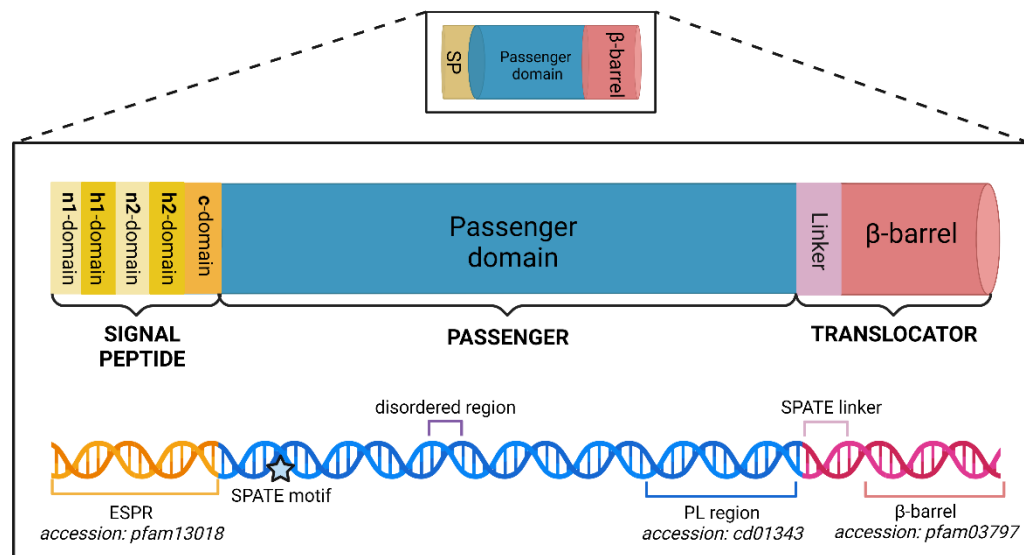


Figure 1.3. A detailed schematic of EspC, used as the prototypical member of the SPATE subfamily of classical ATs. At the top is a mini model used to depict EspC constructs used in this study (see section 2.2.). In the middle, EspC protein domains are subdivided and labelled (see section 1.4.2). At the bottom, EspC is depicted on a DNA sequence level. Not all features present in EspC are applicable to all SPATEs and vice versa (see section 1.4.1.). ESPR – extended signal peptide region; PL– pertactin-like; SPATE – serine proteases of Enterobacteriaceae.

plasmids, chromosomal pathogenicity islands (PAIs) and prophages, but they are classified by their effect on the host cell into Class I - cytotoxic and Class II – immunomodulatory (Table 1.1.) (46, 47, 48). Throughout writing this literature review, a new study has reclassified ATs after a comprehensive phylogenetic analysis by Clarke *et al.* (2022) (49). The 112 functionally characterised ATs comprise 16 homologous groups according to their distance on the phylogenetic tree and functional similarity. Their analysis of proteases coincided somewhat with that of older classification systems, with Class I and II SPATEs spanning Groups 1 and 2 of Cluster A (chymotrypsin-like serine proteases), respectively (49). SPATEs were also associated for the first time with SPATE-like ATs from outside of Enterobacteriaceae (SLATs) that make up Group 3.

Plasmid-encoded	Reference
Sha - exPEC	(50)
EatA - ETEC	(51)
EpeA – EHEC	(52)
EspP – STEC/EHEC/aEPEC	(53, 54)
Hbp – APEC	(55)
Pet – EAEC	(56)
SepA – <i>S. flexneri</i>	(57)
Tsh - APEC	(58)
Chromosome-encoded (PAI)	
EspC – EPEC	(59)
TagB, TagC – APEC	(50)
TleA – ETEC	(60)
Espl – STEC	(61)
Pic – EAEC/ <i>S. flexneri</i>	(62)
Vat - APEC	(63)
Vat-AIEC - AIEC	(64)
Prophage-encoded	
EaaA – commensal <i>E. coli</i>	(65)
EaaC – commensal <i>E. coli</i>	(65)

Table 1.1. Members of Serine Proteases Autotransporters of Enterobacteriaceae don't share a single point of origin on the bacterial chromosome. Although some autotransporters fit into chromosomal PAIs amongst other virulence genes (for example, LEE-encoded virulence genes), others are transferred horizontally (plasmids) or inherited from parts of the phage genome integrated into the bacterial chromosome (prophages). exPEC – extraintestinal pathogenic *E. coli*; APEC – avian pathogenic *E. coli*.

SPATEs cleave a wide range of host cell substrates, summarised by Pokharel *et al.* (2019) and shown in Figure 1.4. (47). SPATEs have recently been implicated in degrading bacterial protein targets (66). Only a few SPATE crystal structures have been solved - EspP (UniProt ID: Q7BSW5), Hbp (ID: O88093) and Pet (ID: O68900), and they remain the basis of proposed T5a secretion models (67, 68, 69).

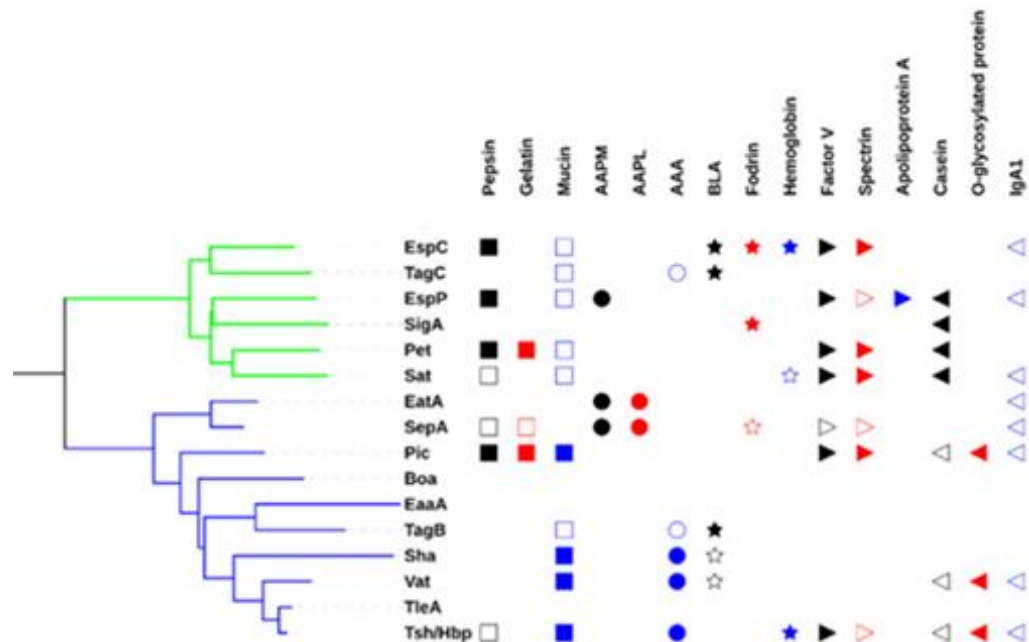


Figure 1.4. Evolutionary relationships between SPATEs according to their passengers and known target substrates. The figure has been adapted from Pokharel *et al.* (2019) (47). Cytotoxic (Class I) SPATEs are in green, while immunomodulatory (Class II) are in blue. Filled symbols show cleaved substrates, unfilled symbols show non-cleavable substrates and empty spots represent untested substrates. AAPM - MeOSuc-Ala-Ala-Pro-Met-pNA; AAPL - Suc-Ala-Ala-Pro-Leu-pNA; AAA - N-Succinyl-Ala-Ala-Ala-p-nitroanilide; BLA - N-Benzoyl-L-arginine 4-nitroanilide.

1.4.1. EspC

The classical AT EspC (EPEC-secreted protein C) is the first protein to be secreted by EPEC (around 60 min into the incubation with IECs) when infecting HeLa IECs (70). The *espC* gene encodes a ca. 140 kDa protein, but the secreted version found in culture supernatants is only ca. 110 kDa, corresponding to the EspC passenger (see Fig. 1.3.). EspC has cytotoxic effects when internalised by mammalian cells; it cleaves a range of host substrates (Fig. 1.4.) and subsequently degrades paxillin and focal adhesion kinase (FAK), causing cell detachment and death (71, 72). It

was also shown to regulate T3SS-mediated pore formation during EPEC infection by degrading T3SS components EspA and EspD (73). EspC is acquired by and prevalent in diarrhoeagenic strains of *E. coli*. It is more readily found in children with diarrhoeal disease than in the asymptomatic cohorts, indicating its importance in pathogenesis (6). A large number of both tEPEC and aEPEC strains possess EspC (74). It has been identified for the first time in ExPEC isolates of human bacteraemia (48).

1.4.2. The signal peptide

As mentioned in section 1.3.1.1., nascent secreted proteins contain a ~25 amino acid long signal peptide (SP) which gets cleaved during IM translocation and directs the pre-protein towards the Sec machinery in an unfolded state. SP sequences have a common organisation consisting of an N-terminal charged region (n), a hydrophobic region (h) and a C-terminal region (c), which includes the cleavage site (19). A few ATs, exclusively those larger than 100 kDa, were found to have a highly conserved N-terminal extension to their SP. This extension included duplicated n and h regions which led to the name 'extended signal peptide regions' (ESPR, Fig. 1.3.) (75, 76). This 24 amino acid long N-terminal extension isn't needed for successful translocation and function of the *E. coli* SPATE Pet (77). Paradoxically, a recent study found that the trimeric autotransporter adhesin (TAA) VtaA (of *Haemophilus parasuis*) cannot cross the IM in mutants lacking ESPR when expressed in *E. coli* (78). While it was initially proposed that the extension prevented the SRP from targeting ATs to Sec co-translationally, it was later proven the cytoplasmic chaperone Trigger Factor prevented SRPs from targeting ATs (79). Studies on numerous classical ATs and their ESPRs found no evidence of its effect on the choice of targeting pathways. Hbp, a member of SPATE, is co-translationally targeted with or without the presence of ESPR (19, 80). More studies are needed to find the evolutionary reason for ESPR and to determine IM targeting pathways of a more significant number of ATs (see section 1.5.).

1.4.3. Passenger

The catalytic S within the GDSGS motif that all SPATES bear in their passenger is determined by strict geometry between the S and nearby asparagine (N) and histidine (H) to form a catalytic triad. Due to the high diversity of substrates cleaved by the SPATE subfamily (see section 1.4.1., Fig. 1.4.), it is impossible to discuss the specific functions of SPATE passengers in detail. Instead, an overview of the passenger domain based on phylogenetic data published will be presented here, with SPATEs evolutionary significance highlighted.

Passengers of 'classical ATs', including SPATEs, are predicted to form β -helices (496 out of 507 ATs investigated) (81). This ability to fold into similar structures might explain how ATs share a secretion mechanism, despite their functional diversity. After analysing 20 SPATE passengers, 173 conserved residues (~1200 residues in total) were found clustered mainly in the first half of the passenger (82). These structural motifs confer substrate specificity, linking SPATE structure to that of chymotrypsin-like serine proteases. Dutta *et al.* (2002) found SPATE passenger domain sequences don't correlate with the substrates they cleave, suggesting many homologous recombination events occurred between the family members (83). When testing for oligopeptide specificity, Dutta *et al.* (2002) also found that the closely related Pet and Sat (Fig. 1.4.) don't share target site specificity, even though they both cleave the same substrate (spectrin). In contrast, the less similar Pic and Sat share aspects of their target site specificity, although they belong to different SPATE classes (Fig. 1.4.) (47, 83). This means SPATEs differ by substrate specificity and oligopeptide specificity; they can cleave the same substrate but leave unique cleavage profiles. The specificity for specific oligopeptide motifs might indicate they likely co-evolved from a horizontally transferred progenitor SPATE, with donor species adapting it to fill their particular virulence needs (84).

An investigation into Pertactin, a classical AT of *Bordetella pertussis*, revealed its passenger folds exceptionally slowly compared to other

protein folding studies (81). This could be a conserved AT feature which prevents premature folding in the periplasm to use sequential folding to drive OM translocation. Interestingly, pertactin-like (PL) passenger regions are conserved in a subset of ATs, but their function is unclear. The region seems to encode a folded structure which overlaps with β -helices found at the C-termini of solved passengers (44). Due to partial sequence overlaps, the term 'PL region' has been used interchangeably with 'stable core' and 'autochaperone', even though these describe structural (81, 85, 86) and functional features, respectively (see below). The inaccuracy of the nomenclature substitution is due to i) the features being identified in only a small number of ATs (so they are often just assumed to be in all ATs), the recent findings that ii) VacA and PrtS passengers display autochaperone function without containing a PL region (87, 88) and that iii) IcsA's stable core does not perform an autochaperone function despite having a PL region (89). Researchers are advised against using the terms synonymously (44).

1.4.3.1. Autochaperone

The term 'autochaperone' was first used to describe a region in the passenger domain of ATs which functions as an obligatory intramolecular chaperone. This protein region can restore passenger folding even when introduced *in trans* (88, 90). Due to some sequence overlap with the PL region, scientists started to use the terms synonymously, but this is now discouraged due to the low number of well-characterised AT autochaperones (ACs) (see section 1.5.2.) (44). Following this criticism, Rojas-Lopez *et al.* (2017) set out to identify AC domains in the widely accepted database of 1523 ATs (91, 92). After performing structural alignments of solved AT crystal structures, the consensus structure was used to identify AC regions in close to 50% of ATs, but EspC wasn't one of them (92). SPATE members with identified AC domains include EspP, Pet, SepA, Pic and Hbp/Tsh (92, 93, 94, 95).

1.4.3.2. Stable core

The C-terminus of some β -helical AT passengers contains the 'stable core', a ~25 kDa structural feature marked by the increased resistance to denaturation and protease digestion (81, 85, 86, 96, 97). Although researchers have proposed the increased stability of this feature promotes efficient secretion across the OM, it doesn't seem to be widespread or the sole mechanism of successful translocation. The efficient secretion of an IcsA homologue in *Yersinia pestis* relies on the global destabilisation of its passenger brought on by 49 nucleotides located at the N-terminus (98).

1.4.4. Translocator

1.4.4.1. Linker

The linker is a universal AT feature connecting the passenger and the β -barrel and is essential for their successful secretion. In the case of ATs whose passengers get cleaved off and released into the extracellular milieu, the cleavage site is often encoded within the linker. Similarly to the PL region, the term 'linker' is used ambiguously, and it is hard to combine all the findings into one clear explanation of its structural and functional significance (44). SPATEs all contain a strictly conserved linker sequence (EVNNLNKRMGDLRD, with NN denoting the cleavage site) which was predicted (and subsequently proved) to fold into an α -helix which extends through the β -barrel and remains there following OM translocation (99, 100, 101, 102, 103). The release of the passenger arises autoproteolytically, with the two asparagines (NN) forming a catalytic dyad. After a change in conformation following OM translocation, the two aspartates are closer and able to interact to trigger cleavage (104). The second N aids the folding of the α -helix, stabilising the β -barrel (101, 103). Some ATs contain disordered (proline-rich) linker sequences upstream of the α -helix, which lack the hydrophobicity required for cooperative folding and therefore don't assume a tertiary

structure (105). Drobnak *et al.* (2014) investigated the prevalence of these regions in 1882 ATs but found only 18.5% contained them, with some predicted disordered regions not even containing prolines (44). Following the same method, 'ESpritz' was used to predict disordered protein regions in the linker of the wild-type EspC sequence (UniProt ID: Q9EZE7), but none was found (Fig. 1.3.) (44, 106).

1.4.4.2. The β -barrel

The C-terminal β -barrel is the most conserved AT feature and is utilised as the primary characterisation tool. Unsurprisingly, it can still be sub-classified due to the ubiquity of ATs in bacterial species and genera (accession numbers: smart00869, pfam03797). All 'classical' AT β -barrels (both solved and predicted) are 12-stranded structures which form a hydrophilic pore in the OM. After translocation, the linker blocks this pore (91, 100, 101, 102, 103, 107). Nevertheless, differences observed in domain arrangement, polypeptide transport-associated (POTRA) domains and oligomerisation states of different β -barrels have been reported (108). Until recently, it was believed the β -barrel autonomously inserts into the OM to facilitate AT secretion, but this has been widely disproved. Due to the fact controversies exist solely around its secretion function and neighbouring protein-protein interactions, the β -barrel will be discussed in more detail in the AT biogenesis subchapter (see section 1.5.).

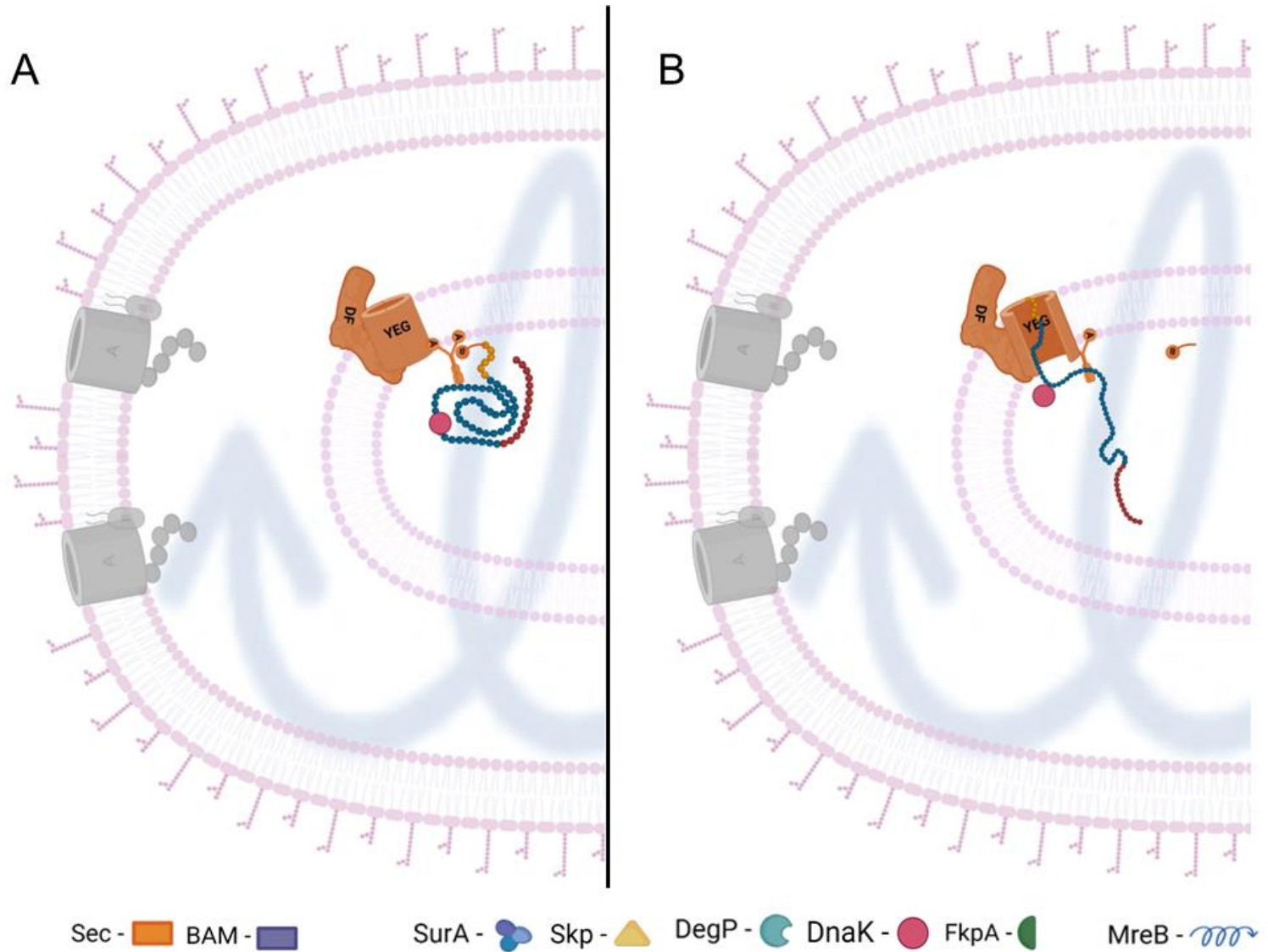
1.5. The current T5aSS secretion model

After an extensive literature review, the following steps of 'classical' secretion are outlined in this subchapter. The cytoplasmic chaperone SecB recognises and binds most nascent AT SPs, delays premature folding and escorts them towards the SecYEG-bound SecA (see section 1.3.1.1., Fig. 1.5.A.). Another cytoplasmic chaperone, DnaK, is essential for the secretion of IcsA & SepA of *S. flexneri* and EmaA of

Aggregatibacter actinomycetemcomitans (23, 109). DnaK was also found to compensate for SecB inactivation partially (Fig. 1.5.A-B.). Upon binding of the AT, SecA triggers the opening of the SecYEG channel and undergoes cycles of ATP hydrolysis to provide energy for N-terminal translocation of the AT across the IM (Fig. 1.5.B.) (21, 24, 25). When the AT stops getting 'threaded' through the SecA binding site, its absence signals SecA to stop hydrolysing ATP until the next nascent polypeptide binds to it (24, 110). On the periplasmic side, SecDF are helping IM translocation by providing the 'pulling' force (111). Although the role of ESPR in deciding IM targeting pathways has been disproven, EspP without ESPR is deficient in OM translocation due to a tendency to misfold in the periplasm (112). Coupled with the finding of an overall decrease in IM translocation in ESPR-maltose binding protein (MBP) fusions (maltose binding protein), it is now thought ESPR plays a hand in slowing down IM export while associated with the Sec translocon (112). Even though the same ESPR-mediated IM stalling has been observed in another *E. coli* SPATE, Pet, it showed no translocation impairment in ESPR deletion mutants (77, 113). Jong *et al.* (2010) have shown the IM insertase YidC is closely associated with the Sec translocon and the ESPR of Hbp. Without it, Hbp and EspC (both SPATEs) accumulate and get degraded by the periplasmic chaperone DegP (114). In contrast, a non-SPATE IcsA doesn't require YidC for its secretion (115). An alternative proposition for the necessity of ESPR to stall IM translocation is that while it's anchored there, it prevents Sec accessory factors (e.g. PpiD chaperone) from clearing the translocon of substrates (19, 116). After being 'ushered' through the SecYEG channel by SecA, the AT stays anchored to the channel by its SP and undergoes c-domain cleavage, leaving the SP behind. This might be how ESPR slows down IM export; while anchored to the SecYEG translocon, it stalls further IM translocation by preventing accessory factors from clearing the SecYEG channel while awaiting recruitment of periplasmic chaperones and cleavage of the SP. This might be limited to SPATE and SLATs, but that's yet to be confirmed.

Figure 1.5. A sequential schematic of 'classical' autotransporter biogenesis in the cytosol and its inner membrane translocation.

The same region of a bacterial cell is magnified and represented in each panel (see Figs 5-8AB). Only proteins active at this stage are shown in colour. (A) After synthesis in the cytosol, the nascent AT with three distinct domains (SP yellow, passenger blue, translocator red) is recognised by SecB and targeted towards the SecYEG-bound SecA. Another chaperone, DnaK, ensures no premature folding takes place. (B) Upon binding SecA, the SecYEG channel opens to allow translocation. SecB releases the AT while SecA undergoes ATP hydrolysis to push the AT across the channel.



The periplasm of Gram-negative bacteria is filled with chaperone molecules performing quality control functions. Proving ATs interact with some of these chaperones sequentially caused the shift from considering T5SS a simplistic secretion system to realising it is a complex and poorly understood mechanism (117). SurA, Skp and DegP chaperones have all been shown to have genetically redundant functions (118). SurA primarily targets outer membrane proteins (OMPs) to the OM, while DegP and Skp help rescue off-pathway proteins or degrade misfolded proteins (119). SurA deletion significantly impacts OM density and integrity, whilst the same is not observed for Skp and DegP mutants (119). Classical ATs EspP, Hbp and IcsA were shown to require the SurA chaperone for successful secretion (120, 121, 122). Moreover, SurA directly interacts with Hbp and EspP's conserved aromatic-X-aromatic motifs located N-terminally in SPATE passengers (43, 121, 122). SurA binding is thought to protect ATs from getting degraded by proteases and target them to the BAM complex (121).

Regarding Skp, scientists have shown it interacts with passengers of EspP & Pet and β -barrels of EspP & NalP (of *Neisseria meningitidis*) (117, 123, 124). Skp mutants are impeded in their ability to secrete EspP & IcsA, but not Hbp & Pet (120, 121, 122, 123, 125). Apart from the general chaperoning function, DegP switches to protease activity at higher temperatures, possibly because it is easier to fix misfolds at lower temperatures (126). DegP interacts directly with EspP (121). DegP mutants are defective in secreting EspP & IcsA but not Hbp (120, 121, 122, 125). Interestingly, pertactin was also shown to require DegP in its biogenesis, but only with its original SP. When its SP was changed to prefer co-translational IM targeting, DegP dependency diminished (127). Another chaperone interaction was captured between EspP and FkpA; a peptidyl-prolyl cis-trans isomerase with a suspected 'timer' function, keeping ATs in a translocation-competent state until they reach the β -barrel assembly machinery (BAM) (128). Recently, the chaperone OsmY was shown to be essential for the biogenesis of AIDA-like ATs (adhesins involved in diffuse adherence), with a suspected role of targeting them to

the Translocation and Assembly Module (TAM) (see below) (129). Finally, IcsA and Pet rely on interactions with VirK, which is thought to protect unfolded passengers in the periplasm safe from proteolysis (123, 130).

Following the initial discovery of periplasmic chaperones interacting with ATs to secrete across the OM, not enough has been done to characterise these further and create a detailed secretion model. Instead, the past decade has seen many studies done only on a handful of ATs, with many differences not addressed. To reconcile some of these findings, I propose the following consensus for classical AT movement (T5aSS) in the periplasm (Fig. 1.6.):

- i) Following IM translocation, while its SP is still anchored to the Sec translocon, the AT passenger domain is accessible to the binding of periplasmic chaperones
- ii) Autoproteolytic cleavage takes place in the c-domain of SPs to release the passenger into the periplasm
- iii) Sequential periplasmic chaperone interactions are required to keep classical ATs translocation-competent, shield them from proteolysis, degrade misfolded proteins and target them to OM secretion complexes (BAM or TAM, see section 1.6.)
 - a. Classical ATs may or may not have evolved motifs in their passengers (or β -barrels), which attract specific quality control chaperones, e.g. the observed amino acid specificity and passenger variations in SPATEs (see section 1.4.3.)
 - b. Following a., studies on SurA, Skp, DegP, FkpA, VirK and their interactions with other AT groups (T5b-f secretion systems) might result in even more confusing and seemingly contrasting findings.

Point iii)a. is inferred from the fact that even classical ATs from the same subfamily show no apparent trend in chaperone dependence: EspP secretion is affected by SurA, Skp and DegP; Hbp secretion depends on

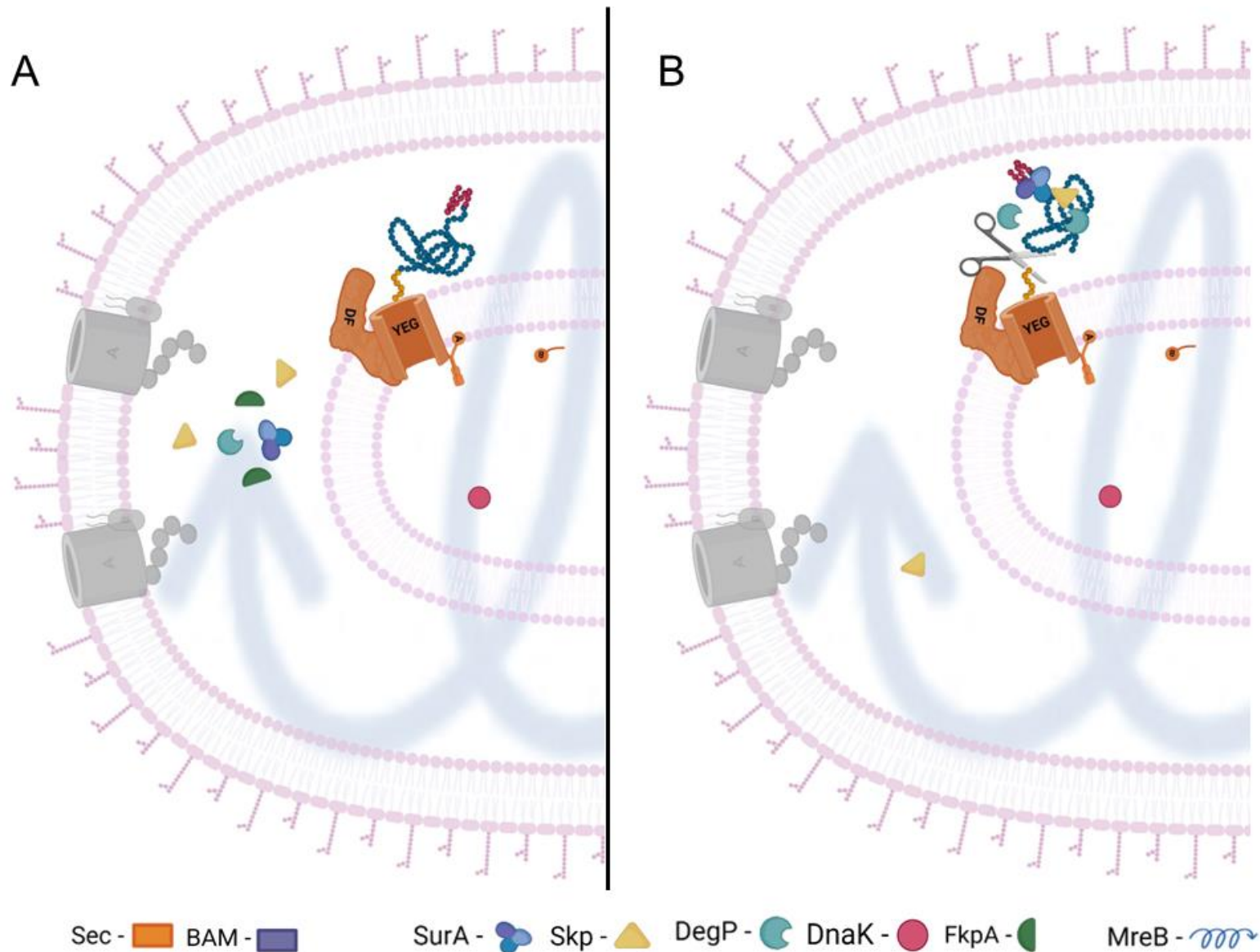
SurA, but not Skp or DegP; Pet interacts with Skp, but the deletion doesn't affect its secretion, etc... Table 1.2. lists the ATs in which chaperone interactions (or dependency) have been observed. It is necessary to study more classical ATs and their chaperone dependency to see if common themes emerge independently of standard AT classifications (to confirm they are accurately grouped). This could be solved by studying classical ATs in both their native and non-native environments to a) determine whether chaperone-specificity is an intrinsic AT feature (did ATs evolve to bind specific chaperones), b) note trends among AT-chaperone interactions when observed in a single model organism (it could also confirm the chaperone pathway is universal or if it differs between species) and c) give insight into differences in T5aSS between bacterial species.

AT NAME	SPECIES	SurA	DegP	DnaK	Skp	FpkA	VirK	DsbA	Tim10	OsmY	HtrA	REFERENCES
IcsA (T5a)	<i>S. flexneri</i>	+	+	+	+							(109, 120, 131, 132)
Pet (T5a)	EAEC						+					(123)
Hbp (T5a)	<i>E. coli</i>	+	+					+				(122, 125)
EspP (T5a)	EHEC	+	+									(43, 121, 133)
YadA (T5c)	<i>Y. enterocolitica</i>	+			+				+			(134, 135)
Pertactin (T5a)	<i>B. pertussis</i>		+									(127)
IgA (T5a)	<i>N. gonorrhoeae</i>					+		+				(136)
NalP (T5a)	<i>N. meningitides</i>				+							(124)
Intimin (T5e)	<i>E. coli</i>	+	+		+			+				(30, 137)
TibA (T5a)	ETEC									+		(129)
EhaA (T5a)	EHEC									+		(129)
AIDA-I (T5a)	<i>E. coli</i>				+							(138)
Hap (T5a)	<i>H. influenzae</i>	?			?						?	(139)
Ag43 (T5a)	<i>E. coli</i>	+								+		(140)

Table 1.2. Periplasmic chaperone – autotransporter protein-protein interactions reported so far. A search has been conducted for all AT research showing chaperone interactions or dependency. Following the AT name, T5a-e denotes the Type 5 subtype (see section 1.3.2.). ‘?’ is shown for Hap because its dependence was measured using a ‘cocktail’ of SurA/Skp/HtrA chaperones instead of testing them separately (139).

Figure 1.6. A sequential schematic of 'classical' autotransporter secretion steps in the periplasm.

The same region of a bacterial cell is magnified and represented in each panel (see Figs 5-8AB). For simplicity, only proteins active in this stage are shown in colour. Following translocation through the SecYEG channel, the signal peptide of the AT remains tethered to the channel. During this time, periplasmic chaperones arrive (A) and bind (B) the AT to keep it in a translocation-competent state and shield it from degradation by proteases. Intramolecular cleavage occurs in the c-domain of the signal peptide to release the chaperone-bound AT passenger into the periplasm (B).

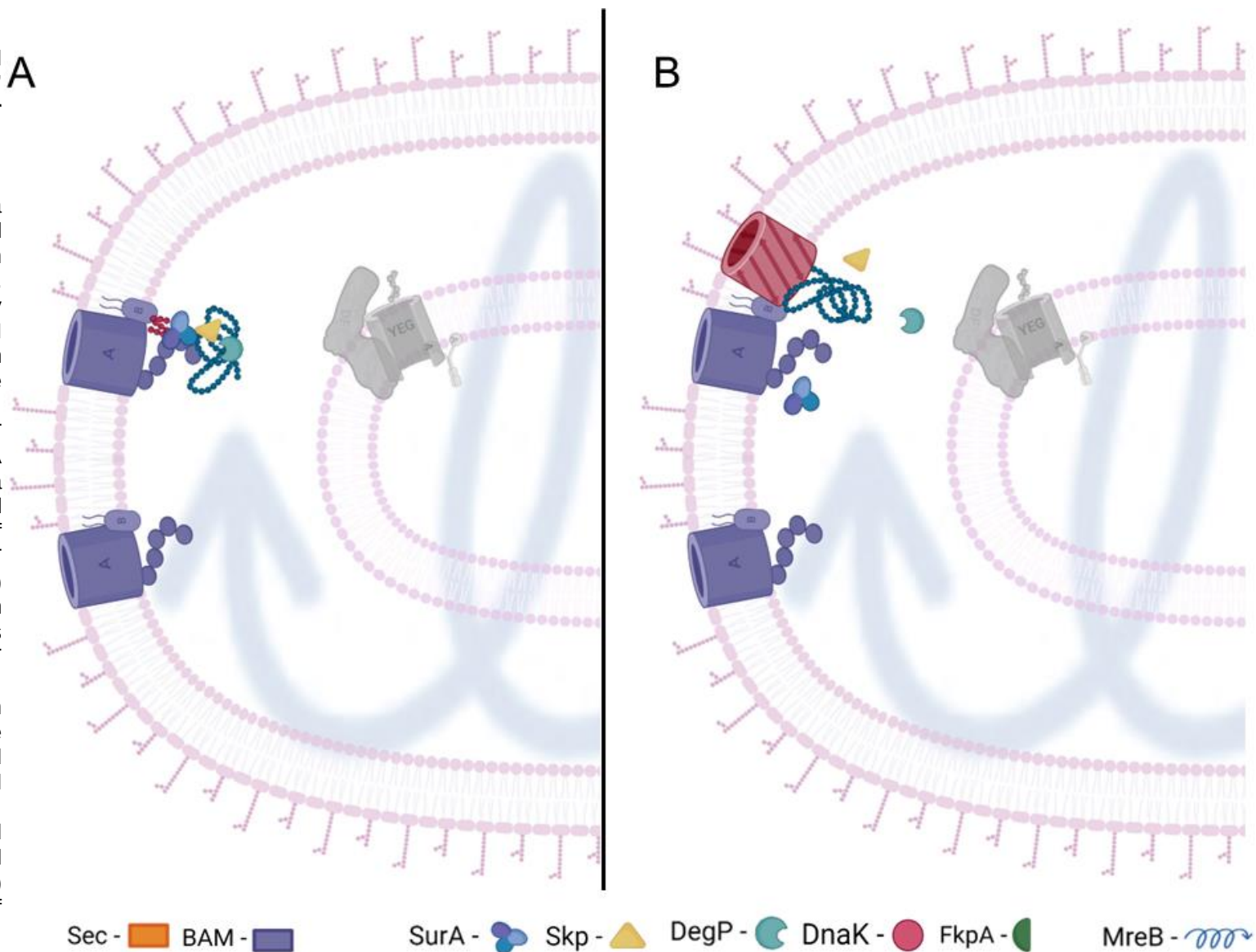


A new era of AT secretion research began with Jain and Goldberg's report that IcsA and SepA interact with the BAM (β -barrel assembly machinery) during their time in the periplasm (141). Coupled with the revealed dependency on periplasmic chaperones, this proved the AT β -barrel is not enough to facilitate AT OM translocation on its own but potentially uses the conserved system developed for outer member proteins (OMPs). In *E. coli*, BAM consists of BamA and four lipoproteins BamB-E which come together to form a hat-like structure (142). BamA (of the Omp85 superfamily) is an OMP with a 16-stranded β -barrel at the C-terminus ('hat crown'), while five POTRA domains dangle off the N-terminus (143). The POTRA domains of BamA and BamB-E form the 'hat's periplasmic brim' (142). BamB binds POTRA2 and POTRA3 domains (P2 and P3, respectively) while BamD forms a subcomplex with BamC and BamE, bridges P1 & P2 with P5, and creates a ring-like docking chamber for chaperones carrying BAM substrates (142). BamA is found to have an outward-open (default) and inward-open (active) conformation. The latter involves its β -strand 1 rotating 90°, opening a lateral gate and remaining exposed to substrate β -barrels (144). BAM involvement in OM translocation has been confirmed in several T5SSs, including 'classical' T5a (43, 117, 122, 141, 145, 146), 'trimeric' T5c (147, 148) and 'inverse' T5e systems (30, 137). Another OM complex was found to be integral for secreting classical ATs Ag43 and EhaA of *E. coli*, although no such role has been noted in SPATEs (122, 149, 150). The Transport and Assembly Module is discussed more in section 1.6.

The linker is the first to fold (forming an α -helix), but it isn't clear if the β -barrel completes folding before reaching BAM (151). A study of the trimeric AT UpaG revealed folding is initiated before passenger secretion, which fits with reports of classical AT secretion being rapid, requiring fast folding (43, 152). BamA recognises the AT-chaperone complex, possibly by its conserved AT β -barrel motif (Fig. 1.7.A.) (153). The docking of the AT-chaperone complex to BamA causes conformational changes in both

Figure 1.7. A sequential schematic of 'classical' autotransporter outer membrane translocation.

The same region of a bacterial cell is magnified and represented in each panel (see Figs 5-8AB). For simplicity, only BamAB of the BAM complex are shown in colour (purple). Once floating in the periplasm, the chaperone-bound AT is recognised by POTRA domains of BamA due to a conserved C-terminal motif (A). The docking of the chaperone-AT complex to BamA (A) activates its inward-open conformation, exposing its β strand 1 and triggers AT release from the complex. The current translocation model suggests that the partially folded translocator binds β strand 1 and continues its folding, creating a hybrid BamA/AT barrel intermediate (not pictured) before budding out of BamA (B).

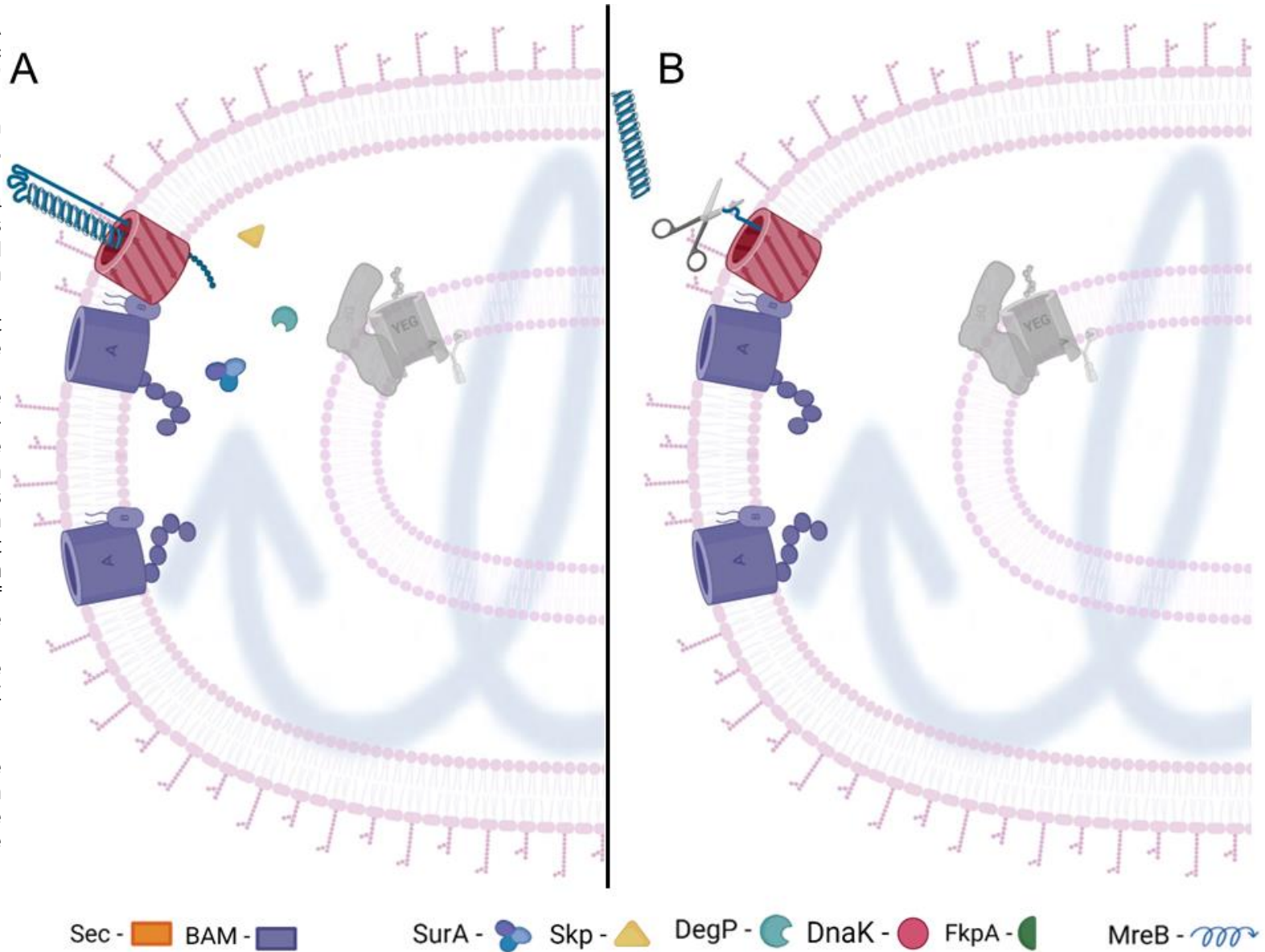


proteins: BamA shifts into its active state (inward-open) and exposes its β strand 1, while SurA triggers the release of the AT substrate (154, 155). How folding is initiated remains unclear, but the currently accepted model is 'budding'; the partially folded β -barrel of the AT binds to the exposed BamA β -strand 1 and continues folding itself, creating an intermediate 'hybrid' barrel (156). When the folding is complete, the AT β -barrel 'buds' out of the complex (Fig. 1.7.B.) (157).

During the past decade, it has been widely accepted that AT passengers translocate across the OM using sequential folding, also known as vectorial folding (81). In this model, the first part of the unfolded passenger (close to the translocator) passively diffuses through the β -barrel. It forms a hairpin structure (Fig. 1.8.A.). Parts of the passenger emerge to the cell surface; it begins to fold and can't go back. A recent study demonstrated that the unfolded AT passenger triggers polymerisation of the 'stable core' (a structural feature found between the passenger and translocator in some ATs, like Pet and Hbp) upon binding to the L5 hydrogen of the first β -hairpin of the translocator (85). This polymerisation results in the secretion and sequential stacking of the passenger until it is a fully formed β -helix outside the cell (Fig. 1.8.A.) (158). In the case of SPATEs, the passenger is cleaved in the linker and released to the extracellular space (Fig. 1.8.B.). Although not much is known about the cleavage step, Peterson *et al.* (2018) have shown the release of the passenger depends on its complete assembly, with misfolded passengers remaining on the surface of the cell for significantly longer (159).

Figure 1.8. A sequential schematic of 'classical' autotransporter passenger domain surface exposure.

The same region of a bacterial cell is magnified and represented in each panel (see Figs 5-8AB). After BAM helps insert the β -barrel into the outer membrane (OM), it is presumed that the still unfolded C-terminus of the passenger domain passively diffuses across the OM. Upon reaching the surface, it starts folding which drives the secretion of the rest of the passenger domain (**A**). Following folding, in the case of SPATE autotransporters, autoproteolytic cleavage occurs in the conserved linker region and releases the passenger into the intercellular space (**B**).



1.6. BAM & TAM dilemma

Phan *et al.* (2021) have recently found that over-production of the BAM has a dramatic impact on the amount of translocated Hbp-antigen fusions, more so than when over-producing periplasmic chaperones (see Table 1.2.) (160). Moreover, they found that mutated Hbp chimaeras accumulate in the periplasm, causing an increase of BAM mRNA and an increase in the amount of DegP produced by the cell (160). Although researchers recognise that the BAM complex is essential to AT translocation (particularly BamA), neither the ‘hybrid barrel’ nor the model for BAM-mediated translocator OM insertion covers all T5SSs, especially trimeric ATs (TAAs) for which no concerted β -barrel insertions have ever been shown. Leo and Linke (2018) raised concerns about the differences in BAM complexity within species that could lead to over-generalising AT secretion models and excluding the whole picture (152, 161, 162). For this author, having reported the above differences even within subgroups of classical ATs (SPATEs), it is entirely possible that T5SSs developed different dependencies on cellular translocation machinery, such as BAM and TAM complexes, through speciation. It was recently shown the ‘classical’ Ag43 could translocate across the OM in *Thermus thermophilus* (belonging to one of the oldest bacterial evolutionary branches), whose BAM complex consists of a single protein – TtOmp85 (BamA homologue) (163). This raises a possibility that functionality pertaining to AT recognition/ β -barrel insertion/sequential folding across the OM is conserved (solely?) in homologues of BamA. Further investigation into TtOmp85 and Ag43 could provide a clearer picture of BAM-mediated translocation and/or give insight into the early evolution of ATs (164).

Unlike the BAM, the Transport and Assembly Module (TAM) machinery spans the periplasm, comprising TamA (exclusive to *Proteobacteria*) at the OM and TamB at the IM (149). While ATs Ag43 (T5a, *E. coli*), EhaA (T5a, EHEC) and intimin (T5e, EPEC/EHEC) require TAM for successful translocation across the OM, it is not needed for OM translocation of TAAs (T5c) (147, 163, 165, 166). TamA is homologous to BamA, apart

from having three (instead of five) POTRA domains which can reach out to interact with TamB. The homology and the finding that BAM can rescue Ag43 secretion raise questions about BAM/TAM cooperation and genetic redundancy (167). Little else is known about TAM, but it's essential for the assembly of fimbriae in *E. coli*, indicating ATs are not its only substrate (168).

1.7. Spatial regulation of bacteria by MreB

The bacterial cytoskeleton is an ordered network of filaments working in concert to maintain their shape, divide, traffic cargo and encompass actin, tubulin and intermediate filament homologues (169). Although MreB (actin homologue) filaments don't assume a higher order structure like actin does in eukaryotes (e.g. webbed networks), both regulate cell shape through self-polymerisation (170). Homologues of *mreB-D* have been identified in rods (*B. subtilis*, *B. cereus*, *E. coli*, *Rickettsia*), curved rods (e.g. *V. cholera*, *C. crescentus*) and helical bacteria (e.g. *C. jejuni*) (170). A consensus has not been reached concerning the size and positioning of MreB filaments (see section 4.1.), but it has been shown they are dynamic - rotating circumferentially (in antiparallel filament pairs) to produce flat ribbons which guide cell wall synthesis (171, 172, 173). Due to the intrinsic property of MreB to recognise parts of the cells with less rigidity (enhanced curvature), it can straighten those regions by guiding the insertion of new cell wall material, along with the rest of the elongasome (comprising MreB-D, RodA, RodZ and PBP2) (174). It is now suggested that MreB could act as a scaffold to provide paths for the localisation and diffusion of membrane-associated proteins, possibly including ATs (175, 176). This fits with the recently discovered role MreB has in creating membrane regions with increased fluidity (RIFs), which, in turn, affect the localisation of membrane proteins (177, 178). RIFs themselves can alter the speed of MreB movement (179).

1.7.1. Subcellular localisation of proteins

Bacterial cells exhibit strict spatial regulation with many essential cell complexes (e.g. chemotaxis, cellular differentiation) and individual proteins shown to localise at specific sites in the cell, mainly at the cell poles of rod-shaped bacteria (180, 181). Polar localisation has been revealed for virulence factor secretion systems in several Gram-negative species (182, 183, 184). A study on the AT IcsA of *S. flexneri* (aids in biofilm formation) showed that polarity doesn't depend on AT domains, hinting at the existence of a conserved cellular targeting machinery (185). One study showed evidence of a helical T4SS distribution in another Gram-negative bacterium, *Agrobacterium tumefaciens* (186). On their way out of the cell, several ATs were found to be localised at the bacterial cell poles (187). This localisation is dependent on i) a functional lipopolysaccharide (LPS) (187), ii) the cytoplasmic chaperone DnaK (109), and iii) a conserved cell division component FtsQ (188). How proteins, including ATs, recognise the poles and how they are delivered to them is not yet fully understood, but suggested theories involve sensing the enhanced curvature of the poles, nucleoid occlusion and recognising mature peptidoglycan (PG) in the absence of new PG synthesis (180, 181, 189). The bacterial cytoskeleton was further implicated in the localisation of proteins when studies showed that defects in bacterial actin homologue MreB synthesis result in the mislocalisation of the type IV pilus retraction ATPase, PilT, away from bacterial poles of *Pseudomonas aeruginosa* (176). Moreover, MreB disturbance affected the localisation of a *Caulobacter crescentus* AT IcsA and several other polar proteins (190, 191). The limitations of localisation studies in general and in ATs will be discussed in more detail in Chapters 3 and 4.

1.8. Techniques for the study of protein interactions

1.8.1. Super-resolution microscopy

Due to the nature of light behaving like waves instead of particles, it is impossible for light 'rays' to illuminate a sharp, bright spot (e.g. fluorescent protein that can be infinitely magnified). Instead, the light waves create a blurry focal spot with a finite size which depends on the light wavelength and the angle at which it intersects with other light waves. In the late 19th century, Ernst Abbe described this diffraction limit, and up until the 1980s, the maximum achievable resolution of a light microscope (the ability to distinguish between two points) was limited to ~250 nm in the lateral directions (x, y) and ~550 nm axially (z). Because objective sizes are finite, collecting all diffracted light from the specimen is impossible, so only low-frequency light is collected. The high-frequency light (the edges and fine details) is not collected, so the image resolution is limited, and further magnification results in blurriness (Fig. 1.9.).

Super-resolution (SR) microscopy is a group term for imaging techniques that surpass the diffraction limit of light microscopy to visualise subcellular components by collecting high-frequency data. Structured Illumination microscopy (SIM) is a technique which uses mathematically deconvolved interference signals (light hitting a grating of known spacing at a calculated angle) to obtain an SR image. SR-SIM uses the Fourier transform to express any given image as a sum of sines and cosines of different amplitudes – i.e. frequency data (see Fig. 1.9.). To collect high-frequency data, SR-SIM illuminates the observed specimen with a grating (light pattern of known frequency), which uses the emerging moiré fringes (secondary patterns observed when two patterns are superimposed) to calculate optically irresolvable structures. SR-SIM image acquisition and its limits are described in Chapter 4.

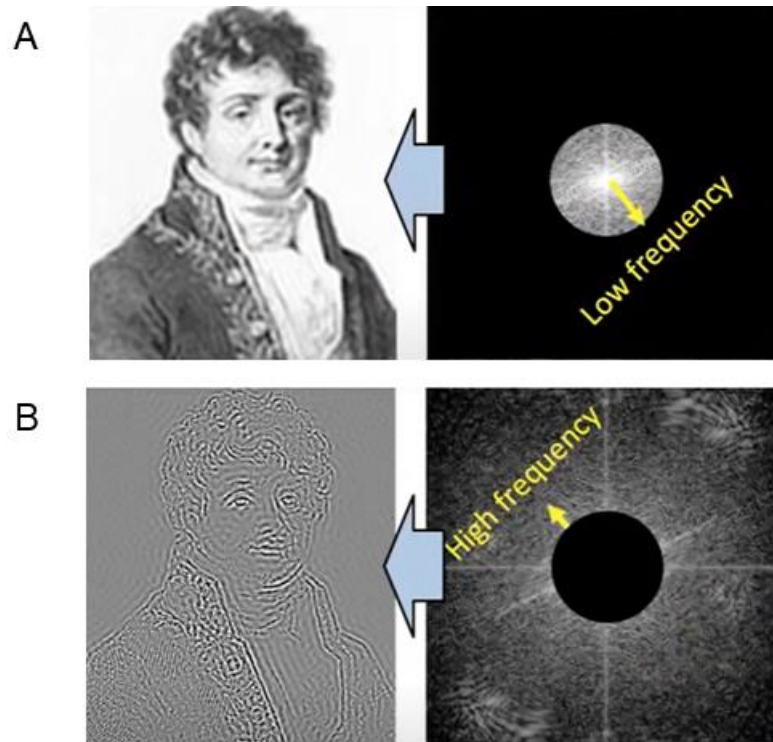


Figure 1.9. An image can be expressed as a sum of sines and cosines of different amplitudes, known as the Fourier transform. A and B contain an image on the left, with the same image represented by the Fourier transform on the right. (A) Light microscopes collect low frequency data (limited by objective size), resulting in recognisable strokes, but no details. (B) The same image is reconstructed using high-frequency data. The fine details, edges and pattern differences are visible. By collecting high-frequency data, it is possible to circumvent the diffraction limit of microscopes. This figure was taken from the lecture “Fourier space” (Bo Huang, iBiology).

1.8.2. Protein labelling techniques for *in vivo* imaging

Surpassing the diffraction limit provided researchers with a chance to resolve single cells. The effect can be enhanced by using chemical dyes that non-specifically bind their target organelles and fluoresce under UV excitation (e.g. DAPI binds DNA and illuminates the nucleus). Only after the discovery of fluorescent proteins (FPs) was it possible to visualise specific proteins and where they are located within the cell. New imaging techniques and fluorescent tags are still being developed, with the best approach depending on the structure (e.g. size and domain function), subcellular localisation (e.g. cytosol, periplasm) and the final destination of the protein of interest (POI) (e.g. secreted extracellularly, OM bound or IM integrated). FPs are a widely used protein tag due to the reliability of their fluorescent signal and photostability. Most originate from aquatic

eukaryotes, meaning the fused FPs are relatively large compared to the bacterial proteins they illuminate. This raises questions about whether FPs accurately show protein localisation in native conditions or cause the recombinant protein to misfold, cluster or lose its functionality (192).

Hybrid probes (e.g. tetracysteine-based FIAsh-EDT₂ labelling) refer to genetically engineered peptide tags in combination with fluorophore-containing dyes. This greatly reduces the chance the tag will affect protein function or interrupt folding since the tag can be as small as six residues in the case of the tetracysteine tag (TC; CCPGCC). The downside to hybrid tags is that the exogenously added dyes can often be cytotoxic, provide a low signal-noise ratio or suffer low membrane permeability (192). Other hybrid probes like SNAP and CLIP are also used, but they are much heavier (~20 kDa), enhancing the risk of function disruption. FIAsh technology is discussed further in Chapter 4.

1.8.3. Extracellular labelling using SpyCatcher technology

The SpyTag-SpyCatcher system was developed from *S. pyogenes* fibronectin-binding protein FbaB. Its domains were split to make a peptide (termed SpyTag) which forms a quick, stable and irreversible bond with its partner (termed SpyCatcher, Fig. 1.10.) (193). Due to its small size (13 amino acids), the SpyTag is an attractive protein tag which can be coupled to a SpyCatcher fused to a fluorescent protein. This has been of interest in detecting and studying proteins at the OM. Since the SpyCatcher-FP fusion is too large to cross the OM, it only labels proteins once they arrive at the cell surface, making this system ideal for visualising ATs (194, 195).

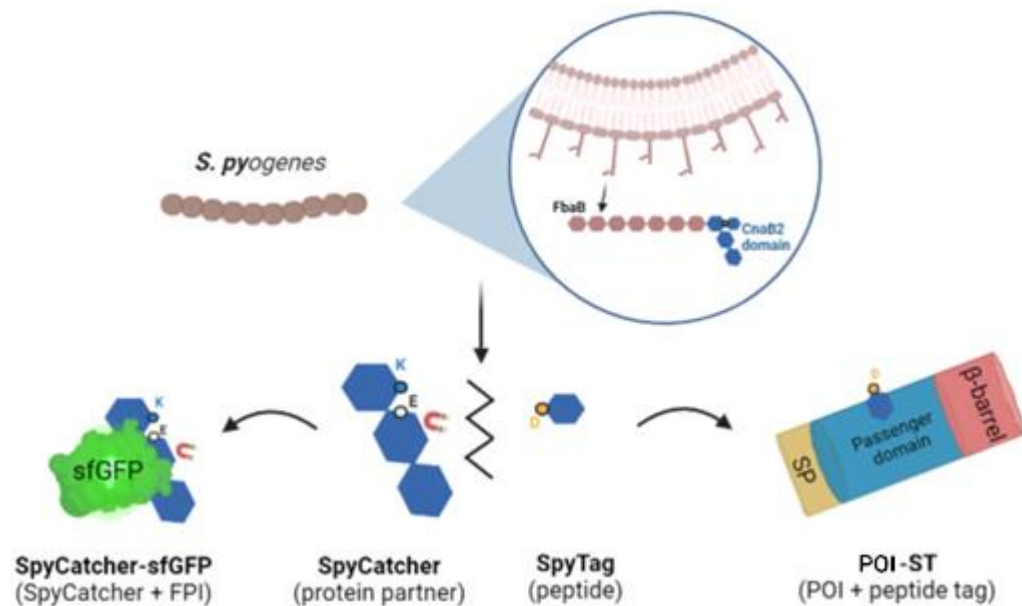


Figure 1.10. The principle of the SpyTag-SpyCatcher system. The OM protein FbaB of *S. pyogenes* contains a domain (CnaB2) with a unique binding property. When physically separated, the two domain pieces readily connect upon re-introduction to form a stable and irreversible bond. The bond forms between lysine (K) and aspartate (D), but it's facilitated and made possible by the neighbouring glutamate (E). The smaller of the two fragments (SpyTag, ST) is only 13 residues long, making it a desirable protein tag. When the tag is inserted into a protein of interest (POI) and its protein partner (SpyCatcher, SC) fused to a fluorescent protein of your choice (FPI), secreted proteins containing the tag can be visualised or quantified at the OM. The magnet next to the glutamate (E) represents the active form of SpyCatcher where E facilitates the formation of the covalent bond between ST and SC.

1.8.4. *In vivo* photo-crosslinking using unnatural amino acids

Apart from visualising protein-protein interactions (PPIs) using the labelling techniques described above, PPIs can be 'frozen' using a variety of chemical approaches (see Chapter 4). Unnatural amino acids (UAAs) are so named because they aren't found in natural polypeptide chains (196). They can be artificially engineered into prokaryote and mammalian cells by reassigning nonsense codons to encode for the desired UAA. This is achieved by the addition of its orthogonal aminoacyl-tRNA synthetase and tRNA partner (aaRS/tRNA_{CUA}) as a plasmid. UAAs have many biotechnological and medical uses, like enhancing polypeptides' biophysical properties, creating antimicrobial peptides and isolating intermediate protein complexes that are sometimes essential for drug development (196, 197). Their use in studying PPIs in bacteria is discussed in Chapter 4.

1.8.5. Antivirulents

Antivirulents are emerging as an alternative way of treating infectious diseases because they inhibit or degrade non-essential factors bacteria use to cause and maintain infections. Since antivirulents target secreted proteins and not particular parts of essential cellular machinery, the bacteria are not killed, but disarmed (198). Without selective pressure to overcome cell death, it is unlikely that antivirulents will cause the emergence of reported antimicrobial resistance mechanisms.

Aurodox (also known as Goldinomycin) is an antimicrobial discovered in the 1970s, isolated from *Streptomyces goldiniensis*. It's grouped with elfamycins - biosynthesis inhibitors targeting the elongation factor Tu (EF-Tu) (199). Aurodox is the N¹-methylated analogue of kirromycin, of the kirromycin family of narrow-spectrum antimicrobials isolated from the *Streptomyces* genus (200, 201). Like its related compounds, it has been shown to bind to EF-Tu and prevent its dissociation from the ribosome. EF-Tu is one of the most abundant proteins in *E. coli*, responsible for transporting aminoacyl-tRNA (aa-tRNA, charged tRNA) to the ribosome for codon-anticodon pairing (202). When it binds guanosine-5'-triphosphate (GTP), it 'switches on', increasing its affinity to bind aa-tRNA. The resulting EF-Tu-GTP-aa-tRNA complex binds the ribosome, triggering conformational changes in the complex, after which an increase in GTPase activity of the complex provides energy for ribosomal decoding and hydrolyses GTP to GDP (guanosine-5'-diphosphate) (199). Upon completion, the complex dissociates, and the process is repeated with the next aa-tRNA. Kirromycins have a common mode of action, consisting of binding to the EF-Tu-GDP complex after GTP hydrolysis, prompting EF-Tu-GDP to remain 'on' and docked to the ribosome. This makes it impossible for the next EF-Tu-GTP-aa-tRNA complex to bind the ribosome, stopping protein synthesis altogether (203). The excitement around kirromycins faded quickly due to their narrow spectrum (a strain or two from *E. faecium*, *S. aureus*, *E. faecalis*, *M. catarrhalis*, *H. influenzae*, *N. gonorrhoeae*). Still, Aurodox has been rediscovered as a T3SS inhibitor (see Chapter 5) (204, 205).

1.9. Hypothesis and Aims

This study aimed to further our understanding of the secretion pathway of the EPEC AT, EspC and extends the findings of Ashawesh (206, 207). Ashawesh *et al.* (2018) found that i) EspC localises to helical patches along the cell, resembling the localisation of MreB and the Sec translocon (208, 209, 210, 211), ii) this localisation pattern is dependent upon the translocator domain of EspC, iii) the distribution is altered when SecA or MreB are disturbed, and iv) that the patchy helices formed by EspC are dynamic and transient, suggesting EspC might be using a cellular scaffold of sorts as a railway to be delivered to secretion sites (cell poles, like other ATs). A direct interaction between EspC and MreB has not been demonstrated, and the mechanistic details remained unclear.

This project set out to:

- I) Develop new fluorescence labelling approaches in *E. coli* suitable for studying subcellular localisation of ATs to validate the existing data generated by imaging ATs fused to mCherry or tagged with the exogenous substrate-based FIAsh technology. This was to be performed using super-resolution microscopy and a novel technology for quantifying protein secretion at the OM, the SpyTag-SpyCatcher system.
- II) Build on fluorescence microscopy data generated by Mahmoud Ashawesh (206, 207) to colocalise EspC with MreB in a single cell, and probe EspC localisation dependency in a broader range of Sec translocon temperature-sensitive (ts) mutants
- III) Crosslink EspC to its neighbouring proteins to confirm its interaction with MreB and to identify new protein-protein interactions to shed light on the molecular components of T5SS pathways.
- IV) Confirm the intertwined regulation reports of T3SS and T5SS by using a T3SS inhibitor Aurodox to study EspC secretion.

2. Methods

2.1. Bacterial strains

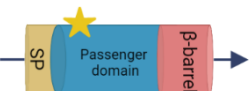
Bacterial strains used in this project are listed in Table 2.1.

Strain	Genotype or description	Reference
MG1655	<i>E. coli</i> F- λ - ilvG- rfb-50 rph-1	(212)
MM52	<i>E. coli</i> temperature-sensitive SecA _{ts} mutant (permissive 30 °C, non-permissive 42 °C); Str ^R	(213)
IQ85	<i>E. coli</i> temperature-sensitive SecY _{ts} mutant (permissive 30 °C, non-permissive 42 °C); Tet ^R	(214)
KJ173	<i>E. coli</i> temperature-sensitive SecD _{ts} mutant (permissive 37 °C, non-permissive 25 °C); Str ^R	(215)
KJ184	<i>E. coli</i> temperature-sensitive SecF _{ts} mutant (permissive 37 °C, non-permissive 25 °C); Str ^R	(215)
EPEC E2348/69	Wild-type EPEC O127:H6 isolated in Taunton (UK) during an outbreak of infantile diarrhoea in 1969	(216)
5-alpha Competent <i>E. coli</i>	fhuA2 Δ (argF-lacZ)U169 phoA glnV44 Φ 80 Δ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17	NEB®
DH10B	<i>E. coli</i> F- mcrA Δ (mrr-hsdRMS-mcrBC) ϕ 80lacZ Δ M15 Δ lacX74 recA1 endA1 araD139 Δ (ara-leu)7697 galU galK λ - rpsL(StrR) nupG	(217)
Turbo Competent <i>E. coli</i>	<i>E. coli</i> F' proA+B+ lacIq Δ lacZM15 / fhuA2 Δ (lac-proAB) glnV galK16 galE15 R(zgb-210::Tn10)TetS endA1 thi-1 Δ (hsdS-mcrB)5	NEB®
NiCo21 (DE3)	<i>E. coli</i> can::CBD fhuA2 [lon] ompT gal (λ DE3) [dcm] arnA::CBD slyD::CBD glmS6Ala Δ hsdS λ DE3 = λ sBamHI Δ EcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δ nin5	NEB®

Table 2.1. Bacterial strains used in this study.

2.2. Plasmids

Plasmid constructs used in this project are listed in Table 2.2.

Plasmid	Construct depiction	Description	Protein size (kDa)	Ref.
pLA33C 1		pBAD33 vector (medium copy number) containing the native <i>espC</i> gene under the control of <i>araBAD</i> promoter; Cm ^R	140	(218)
pLA33C 1-TAG ₉₅₁		pBAD33 hosting the native <i>espC</i> with the amber suppression codon TAG inserted at position 951; under the control of <i>araBAD</i> promoter; Cm ^R	174	(207)
pLA33C 1-ST		pBAD33 vector containing the native <i>espC</i> gene with the SpyTag sequence inserted between S349 and I350 under the control of <i>araBAD</i> promoter; Cm ^R	141.5	This study
pLA33C 1mc		pBAD33 hosting EspC-mCherry fusion (mCherry inserted between L342 and S343) under the control of <i>araBAD</i> promoter; Cm ^R	174	Stephanie Pommier
pLADC1		pBAD18 vector (high copy number) containing the native <i>espC</i> gene under the control of <i>araBAD</i> promoter; Amp ^R	140	(218)
pLADC1 -ST		pBAD18 vector containing the native <i>espC</i> gene with the SpyTag sequence inserted between S349 and I350 under the control of <i>araBAD</i> promoter; Amp ^R	141.5	This study
pLA18C 1mc		pBAD18 vector hosting EspC-mCherry fusion (mCherry inserted between L342 and S343) under the control of <i>araBAD</i> promoter; Amp ^R	174	Stephanie Pommier
pmCherry		pGEMT vector hosting mCherry subcloned from the commercially available pmCherry-C1 (Clontech); Amp ^R	27	Yutivat Vorkunthada
pLA33m Cherry		pBAD33 vector containing the <i>mCherry</i> gene under the control of <i>araBAD</i> promoter; Cm ^R	27	Yutivat Vorkunthada

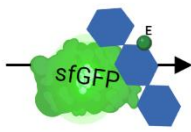
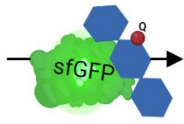
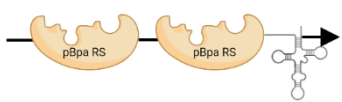
Plasmid	Construct depiction	Description	Protein size (kDa)	Ref.
pLA33m Cherry-TC		pBAD33 vector containing the <i>mCherry</i> gene under the control of <i>araBAD</i> promoter with CCPGCC insertion located after K236; Cm ^R	27.5	This study
pLA33m reB-TC		pBAD33 vector hosting the <i>mreB</i> gene with the CCPGCC motif inserted after G896, under the control of <i>araBAD</i> promoter; Cm ^R	40	(207)
pIBA3-SpyCatcher-sfGFP		His-tagged SpyCatcher protein fused N-terminally to sfGFP. Includes N-terminal His tag for purification and C-terminal StrepII tag for detection; under the control of the <i>tet</i> promoter; Amp ^R	41.3	(195)
pIBA3-SpyCatcherEQ-sfGFP		For the production of the inactive Hist-tagged SpyCatcherEQ protein fused N-terminally to sfGFP. Includes N-terminal His tag for purification and C-terminal StrepII tag for detection; under the control of the <i>tet</i> promoter	41.3	(195)
pULTRA - pBpaRS		pULTRA vector hosting two copies of <i>M.j.</i> p-benzoylphenylalanine reverse synthetase (RS) and its tRNA pair under the control of the <i>lac</i> promoter; Sm ^R	33	(197)

Table 2.2. Plasmids used in this study. Yellow stars represent where SpyTag has been inserted; green probes represent where the tetracysteine tag used for FIAsh-EDT₂ labelling was engineered into, while the E and Q residues in the pIBA3 constructs discern the active from the inactive form of SpyCatcher, respectively.

2.3. Bacterial culture and antibiotics

Unless otherwise stated, all bacterial strains were grown in lysogeny broth (LB; Oxoid). For transformations following site-directed mutagenesis, SOC Outgrowth Medium (New England Biolabs, NEB) was used for recovery. Otherwise, LB was used. Antibiotics were used in the following concentrations: ampicillin (Amp) – 100 µg/mL; chloramphenicol (Cm) – 20 µg/mL; tetracycline (Tc) – 25 µg/mL; spectinomycin (Sm) – 100 µg/mL.

2.4. Growth conditions

Bacterial growth was measured using absorbance (optical density; OD) at 600 nm using the Thermo Biomate 3 Spectrophotometer. Unless otherwise stated, bacterial strains were grown at 37 °C with shaking at 200 rpm. Strain stocks were stored at -80 °C in 40% (v/v) glycerol. Bacterial strains containing MreB-TC or MreB-TC/EspC-mCherry were grown as described in 2.9. When EspC-mCherry was imaged on its own, it was grown as described in 2.4.1, without the temperature switch step (3 h incubation).

2.4.1. Preparation of temperature-sensitive SecYEG mutants

Overnight bacterial cultures (5 mL) of Sec mutants containing the EspC-mCherry (pLA33C1mc) plasmid were diluted to OD₆₀₀ 0.05 in 10 mL, induced with 0.02% L-arabinose, incubated until OD₆₀₀ 0.3 (~2 h) and switched to their non-permissive temperature for another three h to activate their respective mutations. SecA_{ts} and SecY_{ts} mutants were grown at 30 °C and switched to 42 °C. SecD_{ts} and SecF_{ts} mutants were grown at 37 °C and switched to 25 °C (213, 214, 215). Following incubation, the cells were examined using super-resolution Structured Illumination Microscopy (SR-SIM) (see section 2.16.). *E. coli* MG1655 was used as a control cultured in both conditions to observe the effect of EspC secretion and localisation at each non-permissive temperature of 25 °C and 42 °C. Before each experiment, fresh bacteria were plated on LB agar media from the -80 °C stocks to optimise recombinant protein expression (when culturing from older plates, the expression was low, data not shown).

2.5. DNA techniques

2.5.1. Plasmid DNA extraction and purification

Plasmid DNA was extracted using the GenElute™ plasmid miniprep kit (Sigma), following the manufacturer instructions. The DNA was eluted in 50-100 µL elution solution (Sigma). Nanodrop® ND-1000 (Nanodrop Technologies) was used to measure DNA concentrations and evaluate sample purity.

2.5.2. DNA electrophoresis

Gel electrophoresis was undertaken on 1% (w/v) agarose gels containing 1x SYBR® Safe DNA Gel Stain. The electrophoresis was performed at 100 V for 45 min in 1x TAE buffer, and the gels were imaged on Biorad Gel DOC™ XR+ with Image Lab™ Software. The DNA was loaded using 5x GelPilot® Loading Dye (Qiagen), and the Quick-Load® Purple 1kb Plus DNA ladder (NEB) was used to determine the size of DNA fragments.

2.5.3. Site-directed mutagenesis (SDM) polymerase chain reaction (PCR)

Oligonucleotide primers were designed using NEBaseChanger® (NEB®) and synthesised by Eurofins Genomics. Primer sequences synthesised are listed in Table 2.3. NEB® supplied all components for SDM, and the SensoQuest Labcycler was used for the PCR step. Components and thermocycling conditions are listed in Table 2.4. Following amplification, the PCR products were visualised by loading 2 µL of the product on 1% agarose gels (100 V, 45 min). After amplification, the PCR products were processed using KLD or PNK methods described in 2.5.4. and 2.5.5., followed by chemical transformation (2.5.9.). Mutagenesis was validated and confirmed by Sanger sequencing (Genewiz).

Primer label	Primer sequence	Description
pIBA3SpyCatcher	ggtttatcaagtgagcaagggtca	Sequencing primer used for checking the presence of E77 > Q substitution in the SpyCatcher-sfGFP constructs.
MS23fw	ccccgactactgaagctgt	Forward sequencing primer used to check for successful SpyTag insertion between S587 and I588 in EspC-mCherry (pBAD33 and pBAD18 constructs).
MS1213fw	tggtgtggagatcaatgggtg	Forward sequencing primer used to check for successful SpyTag insertion between S348 and I349 in EspC (pBAD33 and pBAD18 constructs).
MS213bw	cattcaatgtacctgcacca	Reverse sequencing primer used to check for successful SpyTag insertion between S587 and I588 in EspC and EspC-mCherry (pBAD33 and pBAD18 constructs);.
MSSP	agttatgaagaaagccgctcg	Forward sequencing primer used to check for successful SpyTag insertion between D59 and N60 of EspC and EspC-mCherry constructs.
MS1	ggctgttgcgcctacaacgtcaacatcaag	SDM primer pair used to insert CCPGCC into pmCherry between positions P194 and G195.
MS2	ggggcaacacagctgcacgggcttctt	
MS3	tggtgccccggcgcctacaacgtc	Primer pair used to insert the first two cysteines into pmCherry before P194. Used instead of MS1/2.
MS4	cagctgcacgggcttcttg	
MS5	tggtgcgccctacaacgtcaacatcaagttg	Primer pair used to insert CC into pmCherry after G195. Used after primer pair MS3/4 successfully integrated the first two cysteines.
MS6	gccggggcaacacagctg	
MS10	ggctgttgacccggcgcatggacgag	SDM primer pair used to insert CCPGCC into mCherry between S226 and T227 (pBAD33 construct).
MS11	ggggcaacaggagtggcgccctcgcc	
MS12	cgccctacaagccgacgaagat taatttcgacaatgacgtaaac	SDM primer pair used to insert SpyTag into EspC and EspC-mCherry between S587 and I588, in the middle of the passenger domain (pBAD33 and pBAD18 constructs).
MS13	tctaccattactatgtgtgcactt ccaccaccactaaaac	
MS14	cgccctacaagccgacgaagctaaatatt gataatgtatgggctag	SDM primer pair used to insert SpyTag into EspC and EspC-mCherry between Q55 and L56, at the beginning of the passenger domain (pBAD33 and pBAD18 constructs).
MS15	tctaccattactatgtgtgcttgagcagcct gagatgc	
MS16	ggctgctgcgtcgacctgcaggcatgc	SDM primer pair used to insert CCPGCC into mCherry at the C-terminus of mCherry, after K236 (pBAD33 construct).
MS17	cgggcagcactgttacagctcgccatgc	
MS18	catcaccatttgaaaaaatttcgtggc	SDM primer pair used to insert 6x His into MreB at the N-terminus of MreB after the start codon (not used, antibody ordered).
MS19	gtgatgggtcatagatctattcgctagc	
MS20	cgccctacaagccgacgaag aatgtatgggctagagattatttag	SDM primer pair used to insert SpyTag into EspC and EspC-mCherry between

Primer label	Primer sequence	Description
MS21	tctaccattactatgtgtgc atcaatatttagtgagcagc	D59 and N60, at the beginning of the passenger domain
MS22	aatgtatgggctagagattatttag	Hi-Fi DNA Assembly primer pair used to amplify EspC and EspC-mCherry fusions from N60 to D59 for SpyTag ligation.
MS23	atcaatatttagtgagcagc	
MS24	ctgctcaactaaatattgatgcacacatag taatggtagacg	
MS25	taatctctagcccatacattcttcgctggctt gtaggc	Hi-Fi DNA Assembly primer pair used to ligate the SpyTag fragment between D59 and N69 of <i>espC</i> .
MS26	gcacacatagtaatggtagacg	Hi-Fi DNA Assembly primer pair used to amplify the SpyTag fragment from an existing ST strain.
MS27	cttcgctggcttgtaggc	
rtMS1	tagtgcagtcagaaagcagtt	The qPCR primer pair used to amplify <i>espC</i> in EPEC E2348/69 (219).
rtMS2	agttttcctgttgctgtatgcc	
rtMS3	tgcaagtcgaacggtaacag	The qPCR primer pair used to amplify <i>rrnB</i> (16S rRNA). Used as the endogenous control in qPCR reactions (220).
rtMS4	agttatccccctccatcagg	

Table 2.3. Oligonucleotide primers used for PCR amplification and site-directed mutagenesis. The primers used for sequencing were generated using Primer3; NEBaseChanger was used to design SDM primers, and NEBuilder was used for Hi-Fi DNA assembly.

2.5.4. Kinase, ligase & *DpnI* (KLD) treatment

The KLD enzyme mix (NEB®) was applied after SDM PCR to remove template DNA and circularise the product to maximise the efficiency of SDM. The mixture was prepared as described in Table 2.5., incubated at room temperature for 5 min and placed on ice to be used in the transformation. Following KLD treatment, due to the high salt content in the KLD buffer causing low transformation efficiency using the electroporator (observed by monitoring the time constant in ms), a simple ‘dialysis’ step was performed to reduce the ion content. A 5 µL droplet of

the KLD mixture was pipetted onto a Whatman® Regenerated Cellulose Membrane Filter Circle floating on nuclease-free water in a petri dish. After leaving the droplet on the membrane for 45 mins, ~10 µL of the filtered product was used in subsequent electroporation.

Component	50 µL reaction	Final concentration
5x Q5 Reaction Buffer	10 µL	1x
10 mM dNTPs	1 µL	200 µM
10 µM Forward Primer	2.5 µL	0.5 µM
10 µM Reverse Primer	2.5 µL	0.5 µM
Template DNA	1-25 ng	<1000 ng
Q5 High-Fidelity DNA Polymerase	0.5 µL	0.02 U/µL
Nuclease-free water	to 50 µL	
Cycle stage	Temperature	Length
Initial denaturation	98 °C	30 sec
25 - 35 cycles	98 °C	5 sec
	60-72 °C	30 sec
	72 °C	30 sec per kb plasmid
	72 °C	2 min
Final extension	72 °C	

Table 2.4. Site-directed mutagenesis reaction components and thermocycling conditions used for insertions. Troubleshooting and optimisation were done using the Q5 Hi-Fidelity SDM kit (NEB®) to tailor the reactions to the plasmids. dNTP - deoxynucleotide triphosphate.

Component	Volume	Final concentration
Unpurified PCR Product	1 µL	
2x KLD Reaction Buffer	5 µL	1x
10x KLD Enzyme Mix	1 µL	1x
Nuclease-free water	3 µL	

Table 2.5. KLD mixture recipe.

2.5.5. T4 Polynucleotide Kinase (PNK) and ligase treatment

Due to the low transformation efficiency observed when using KLD treatment on SpyTag mutants, T4 PNK and ligase were used instead. Following amplification (see section 2.5.3.), the Wizard® SV Gel and PCR Clean-Up System (Promega) was used to purify the PCR product and elute it in 15 µL nuclease-free water. The polynucleotide kinase reaction was assembled on ice (see step 1 in Table 2.6.) and incubated for 60 min at 37 °C. The reaction was stopped by heat-inactivating the sample for 20 min at 65 °C. The resulting T4 PNK product was cleaned up using Wizard® to prepare for the ligation, and its DNA concentration was measured. The ligation reaction was assembled on ice (see step 5 in Table 2.6.) and left to incubate overnight at 16 °C. The reaction was stopped by heat-inactivating the sample for 10 min at 65 °C, after which 5 µL ligation was used in the transformation (see section 2.5.9.).

Step	Component	50 µL reaction
1	T4 PNK	1 µL
	10x T4 PNK Reaction Buffer	5 µL
	Purified PCR product	100 ng
	Nuclease-free water	to 50 µL
2	Incubation at 37 °C for 60 min	
3	Heat-inactivation at 65 °C for 20 min	
4	PCR clean-up of T4 PNK product	
5	T4 DNA Ligase	1 µL
	10x T4 DNA Ligase Buffer	2 µL
	Purified T4 PNK product	25 ng
	Nuclease-free water	to 50 µL
6	Incubation at 16 °C for 16 h	
7	Heat-inactivation at 65 °C for 10 min	
8	Transformation using 5 µL ligated product	

Table 2.6. Post-PCR processing steps used to increase the transformation efficiency of mutants generated by SDM.

2.5.6. DNA assembly

The NEBuilder HiFi DNA Assembly kit (NEB®) was used to ligate the SpyTag (ST) fragment amplified from an existing ST strain to EspC vectors (primer pair MS24-25, see Table 2.3.). Primers MS22-23 were designed using NEBuilder and used to amplify backbone fragments of EspC and EspC-mCherry (Table 2.3.). Amplifying three fragments (pLA33C1, pLA33C1mc and SpyTag) was undertaken using the reagents and conditions listed in Table 2.4. After amplification, the fragments were cleaned using the Wizard® SV Gel PCR Clean-Up System (Promega). The assembly reaction was set up using the Hi-Fi kit in a 5:1 ratio (insert : vector). The samples were ligated in a thermocycler at 50 °C for 15 min, placed on ice, and transformed into Turbo competent *E. coli* (NEB®) using chemical transformation (see section 2.5.9.). Colony PCR was performed on the transformants using the primer pair MS24-25 with the GoTaq® Hot Start Green Master Mix (Promega) using manufacturer instructions. Part of a transformant colony was resuspended in 50 µL NF water and lysed in a thermocycler for 5 min at 95 °C (Table 2.7). SpyTag insertion was verified by electrophoresis of the PCR products on 4% agarose gels for 30 min at 100 V at 4 °C, using a 100 bp ladder (NEB®, see section 2.5.2.).

Component	50 µL reaction	Final concentration
GoTaq® Hot Start Green Master Mix	10 µL	1x
10 µM Forward Primer	2.5 µL	0.5 µM
10 µM Reverse Primer	2.5 µL	0.5 µM
Template DNA	5 µL	
Nuclease-free water	to 50 µL	
Cycle stage	Temperature	Length
Initial denaturation	95 °C	2 min
	95 °C	10 sec
25 - 35 cycles	63.5 °C	20 sec
	72 °C	4 min
Final extension	72 °C	5 min

Table 2.7. Reaction components and thermocycling conditions used for colony PCR.

2.5.7. Preparation of electrocompetent *E. coli* cells

One mL of *E. coli* MG1655 overnight culture was added to a 99 mL flask with fresh LB and incubated at 37 °C until it reached 0.35-0.4 OD₆₀₀ (~2-3 h). The rest of the protocol was performed on ice. The flask was placed on ice for 15 min and swirled occasionally. Two 50 mL Falcon tubes, sterile dH₂O and 10% glycerol, were chilled on ice. The flask was divided into two Falcon tubes and centrifuged at 3000 rcf for 20 min at 4 °C. The supernatant was discarded, and cells were resuspended with 20 mL of ice-cold dH₂O. The centrifuge step was repeated, and cells were resuspended with 10 mL ice-cold dH₂O. The centrifuge step was repeated, and cells were resuspended with 10 mL 10% glycerol. The two Falcon tubes were combined to make one containing 20 mL 10% glycerol. The centrifuge step was repeated, the supernatant discarded, and cells resuspended with 500 µL 10% glycerol. Aliquots of 100 µL were placed into pre-chilled Eppendorf tubes and stored at -80 °C.

2.5.8. Electroporation

Eppendorf tubes (one per reaction) were filled with 950 µL LB/SOC and pre-warmed in an incubator at 37 °C, along with agar plates. All electroporation reagents were held on ice: 2 mm cuvettes, extracted plasmid DNA and electrocompetent cells. 1-3 µL of plasmid DNA was pipetted into the cuvette. Fifty µL of electrocompetent cells were added to the cuvette, which was dried and placed into the electroporator. Negative controls did not contain DNA and were run for each antibiotic resistance marker in the transformation. Electroporation was run on the 'Ec2' program (V = 2.5 kV) of the MicroPulser Electroporator (Biorad). After the reaction, the pre-warmed media was quickly mixed into the electrocompetent cells and placed for recovery at 37 °C for 1 hour. Pre-warmed plates were used to plate 100 µL of the reaction and incubated overnight. Successful transformants were re-plated, grown, and their DNA extracted for sequence verification and storage.

2.5.9. Chemical transformation

Chemical transformations were performed when electroporation following SDM PCR proved unsuccessful. All reagents were held on ice, and 950 μ L of SOC Outgrowth Media (NEB®) was pre-warmed at 37 °C, along with the agar plates. A tube of Turbo competent *E. coli* (NEB®) was thawed on ice. Five μ L of ligated PCR product was added to the competent cells, mixed by flicking and placed on ice for 30 min. Heat shock was performed for 90 s at 42 °C, after which the cells were put back on the ice for 5 min. The pre-warmed media was added to the reaction, and the cells were placed on recovery at 37 °C with shaking (200 rpm). Following recovery, 100 μ L and 150 μ L were plated onto agar plates and incubated overnight.

2.6. Protein techniques

2.6.1. Whole-cell protein preparation

E. coli cells were routinely grown and induced in a way appropriate to the method used (see sections 2.8.4. and 2.15.). The optical density was measured (see section 2.4.), and the volume was normalised across samples and the controls used for protein analysis. The samples were centrifuged for 5 min at 5000 xg and resuspended in 50 μ L 1x phosphate-buffered saline (PBS). 50 μ L of 2x SDS-PAGE loading buffer was added and mixed thoroughly (100 mM Tris-HCl, pH 6.8, 200 mM β -mercaptoethanol, 4% (w/v) sodium dodecyl sulfate (SDS), 0.2% (w/v) bromophenol blue, 20% (v/v) sterile glycerol). The samples were boiled (with intermittent mixing) for 10 min at 95 °C, after which they were centrifuged for 5 min at 16 000 xg to remove cellular debris. Twenty-five μ L was used to load onto SDS-PAGE gels.

2.6.2. SDS-PAGE electrophoresis

Standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE was performed to separate proteins analysed according to size. The recipes used to prepare the gels are listed in Table 2.8. unless stated otherwise. Once prepared and loaded onto the gel, the gels were placed inside the Bio-Rad Mini-Protein® Tetra Cells system and filled with 1x running buffer. The electrophoresis was run using the PowerPac™ HC (Bio-Rad) for 20 min at 70 V, and the current was subsequently increased to 150 V until the loading dye migrated out of the gel. Following electrophoresis, the gels were stained with Coomassie (1.2 g/L Coomassie, 50% methanol, 10% acetic acid) for 30 min at room temperature or analysed using Western Blot (see section 2.8.3.). Stained gels were destained overnight in 50% methanol and 10% acetic acid before they were imaged on the Gel Doc™ XR+ Molecular Imager® (Bio-Rad).

Component	Recipe
Stacking buffer	1 M Tris-HCl, 0.4% (w/v) SDS, pH 6.8
Separating buffer	1.5 M Tris-HCl, 0.4% (w/v) SDS, pH 8.8
Ammonium persulfate (APS)	10% in dH ₂ O
10x running buffer	250 mM Tris, 1.92 M glycine, 35 mM SDS
2x SDS-PAGE loading buffer	100 mM Tris-HCl, pH 6.8, 200 mM β-mercaptoethanol, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) sterile glycerol
Protein ladder	Spectra™ Multicolor High Range Protein Ladder (Thermoscientific)
5% stacking gel (makes two gels)	1 mL Stacking buffer, 723 μL dH ₂ O, 3 mL acrylamide, 50 μL 10% APS, 5 μL tetramethylethylenediamine (TEMED)
12% separating gel (makes two gels)	2.5 mL Separating buffer, 4.44 mL water, 3 mL acrylamide, 150 μL 10% APS, 15 μL TEMED

Table 2.8. Recipes used to prepare reagents for SDS-PAGE.

2.6.3. Western Blot

Following the SDS-PAGE electrophoresis step, the gel was transferred to a nitrocellulose membrane via a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad) filled with 1x Tris-glycine transfer buffer. The transfer was performed at 4 °C for 60 min at 150 mA. Blocking solution (milk) was added to prevent non-specific antibody binding, and the membrane was incubated overnight at 4 °C with shaking. After discarding the blocking solution, the primary antibody was added, and the membrane was incubated for a further 60 min. 1x TBST was used to wash the membrane 3x for 5 min before the secondary antibody was added and shaken for another 60 min. The membrane was rewashed 6x for 5 min with 1x TBST. Lastly, the membrane was treated with Amersham ECL Start Western Blotting Detection Reagent (GE Healthcare) and developed on Amersham Hyperfilm™ chemiluminescence film (GE Healthcare) following the manufacturer's instructions. Reagents used to perform the blot and desirable antibody dilutions are listed in Table 2.9.

Component	Recipe
10x Tris-glycine transfer buffer	250 mM Tris, 1.92 M glycine
1x Tris-glycine transfer buffer	25 mM Tris, 192 mM glycine, 20% methanol
10x Tris-buffered saline (TBS) pH 7.5	200 mM Tris, 1.5 M NaCl
1x Tris-buffered saline Tween® 20 (TBST)	20 mM Tris, 150 mM NaCl, 0.1% (w/v) Tween® 20
Blocking solution (50 mL)	5% (w/v) dried skimmed milk powder, 0.5 (v/v) TBST
α-EspC	1:1000 in blocking solution (221)
α-dsRed	1:10000 in blocking solution (Source Bioscience)
α-MreB	1:10000 in blocking solution (Stratech)
Secondary antibody: Anti-rabbit IgG – peroxidase antibody produced in goat	1:10000 in blocking solution (Sigma)

Table 2.9. Recipes for reagents used in Western blotting.

2.6.4. Nickel Immobilised Metal Affinity Chromatography (Ni-IMAC) protein purification of SpyCatcher proteins

All reagents and their compositions are listed in Table 2.9. The plasmids encoding SpyCatcher WT (pIBA3-SpyCatcher-sfGFP) and SpyCatcher EQ (pIBA3-SpyCatcherEQ-sfGFP) were kindly gifted to us by Dr Jack Leo from Nottingham Trent University (pIBA3-SpyCatcher-sfGFP Addgene plasmid #107420; pIBA3-SpyCatcherEQ-sfGFP Addgene plasmid #107421). Both proteins were purified using the same method described here. The plasmid was freshly transformed into NiCo21 (DE3) cells using 0.5 μ L plasmid DNA and incubated overnight at 37 °C.

Component	Recipe
1.1 L low-salt LB	10 g tryptone, 5 g yeast extract, 5 g NaCl, dH ₂ O; autoclave
Anhydrotetracycline (AHTC)	100 μ g/mL in 50% ethanol
1 L 1x PBS, pH 7.4	137 mM NaCl, 2.7 mM KCl, 10 mM Na ₂ HPO ₄ , and 1.8 mM KH ₂ PO ₄ ; autoclave
disodium phosphate	1M; autoclave
monosodium phosphate	1M; autoclave
500 mL 100 mM phosphate buffer, pH 7.4	38.7 mL disodium phosphate, 22.6 mL monosodium phosphate, 438.7 mL dH ₂ O; autoclave
Nickel-nitriloacetic acid (Ni-NTA) lysis buffer, pH 7.4	20 mM phosphate buffer, 50 mM NaCl, 20 mM imidazole; autoclave
Ni-NTA equilibration buffer, pH 7.4	20 mM phosphate buffer, 200 mM NaCl, 20 mM imidazole; autoclave
Ni-NTA elution buffer, pH 7.4	20 mM phosphate buffer, 200 mM NaCl, 500 mM imidazole; autoclave
dH ₂ O	autoclave
20% ethanol	0.2 μ m filter in the vacuum pump to degas
Charging buffer	0.1 M NiCl

Table 2.10. Reagents used to purify SpyCatcher WT/EQ using Ni-IMAC.

A single transformant was used to inoculate two 10 mL Falcon tubes containing LB and 100 µg/mL ampicillin and incubated overnight at 30 °C. Two 2 L conical flasks containing 500 mL low-salt LB and 100 µg/mL ampicillin were used to grow day cultures from OD₆₀₀ 0.05 and incubated at 30 °C at 180 rpm until OD₆₀₀ 0.6. The inducer anhydrotetracycline (AHTC) was added at a final concentration of 0.1 µg/mL, and the flasks were incubated for an additional four h. The cells were harvested for 15 min at 3100 xg, the supernatant discarded, and the pellet stored overnight at -80 °C. The pellets were resuspended in lysis buffer (Table 2.10.) and treated with the complete Mini, EDTA-free protease inhibitor (Sigma). Cell lysis was performed using sonication with a longitudinal sound-wave amplitude of 15 for 6 min (15-sec pulse, 15-sec pause). A 100 µL lysate aliquot was collected for SDS-PAGE analysis. The rest of the lysates were centrifuged for 30 min at 20000 xg and filtered through 0.2 µL syringe filters (Thermofisher). A benchtop peristaltic pump was used to charge, prepare and load the column, as shown in Table 2.10. Collected aliquots were analysed on SDS-PAGE by mixing 10 µL aliquot with 10 µL 2x SDS-PAGE loading buffer (see section 2.8.2.).

PHASE	Reagent in pump (column volume – CV)	Pump flow rate	Collect aliquot
CHARGE	1 CV 0.1 M NiCl	2 mL/min	x
PREP	10 CV dH ₂ O	5 mL/min	x
	3 CV Ni-NTA equilibration buffer	5 mL/min	x
	3 CV Ni-NTA elution buffer	5 mL/min	x
	5 CV Ni-NTA equilibration buffer	5 mL/min	x
LOAD	Filtered protein lysate	2 mL/min	✓
WASH	5 CV Ni-NTA equilibration buffer	5 mL/min	✓
ELUTE	SpyCatcher WT: 2 CV 40% Ni-NTA elution buffer	2 mL/min	✓
	SpyCatcher EQ: 2 CV 60% Ni-NTA elution buffer	2 mL/min	✓

Table 2.11. Ni-IMAC protein purification procedure using a benchtop peristaltic pump. The collected aliquots were analysed by SDS-PAGE to verify column specificity and sample purity.

Eluted proteins were dialysed in darkness overnight at 4 °C in SpectraPor® membrane tubing (Spectrum™, 132724) and placed in 3 L 1x PBS with 5% glycerol with a stirring bead. Vivaspin® Turbo 15 centrifugal concentrator (Sartorius) was used to concentrate the proteins to ~ 9 mg/mL. Protein concentrations were measured using Nanodrop® ND-1000 (Nanodrop Technologies).

2.7. SpyTag/SpyCatcher experiments

SpyTag/SpyCatcher technology was utilised in several ways. The SpyTag (AHIVMVDAYKPTK) was engineered into EspC and EspC-mCherry constructs, described in sections 2.6. and 2.6.2. (193). For experiments investigating the secretion ability of EspC-mCherry, see section 2.14., while super-resolution microscopy using EspC-ST is described in section 2.12.

2.8. Quantification of EspC at the OM

This method has been adapted from Chauhan *et al.* (195). Due to sharing purified SpyCatcher stocks with a colleague, the volume of SpyCatcher added was decreased from 20 µL to 10 µL as no difference was observed in fluorescence quantification (data not shown). EspC_{ST} (pLA33C1_{ST}) was grown overnight in *E. coli* MG1655, as described in sections 2.3. and 2.4. A non-ST strain was used as the negative control and treated the same way. Day cultures of three biological replicates were inoculated to OD₆₀₀ 0.05 in 10 mL LB containing 0.02% arabinose and incubated for 3 h. The cells were harvested corresponding to 1.0 OD₆₀₀ and resuspended in 1 mL 1x PBS. The cells were treated with 10 µL SpyCatcher WT (9.66 mg/mL stock) for 60 min at room temperature. The cells were washed with 1x PBS three times to remove excess SpyCatcher, and the cells were resuspended in 200 µL PBS. A plate reader was used to measure the fluorescence (absorbance – 485 nm, emission 535 nm) on a black 96-well polystyrene plate (655096, Greiner).

2.9. Detection of EspC in supernatants following incubation with Aurodox

This method has been adapted from (205), while instructions from (73) were used to optimise EspC expression in wild-type EPEC E2348/69. An overnight E2348/69 was grown in 5 mL LB with no antibiotic selection as described in 2.4. Commercially available Aurodox (27584, Cayman Chemicals) was prepared by dissolving it in dimethyl sulfoxide (DMSO) to make 1 mg/mL stock (levels of DMSO in all experiments were < 0.5%). Overnights were used to prepare seven tubes with 5 mL DMEM each corresponding to the final OD₆₀₀ of 0.05. With the first Falcon tube being the control, no Aurodox was added, while 1.25 µg/mL, 2.5 µg/mL, 3.75 µg/mL, 5 µg/mL, 7.5 µg/mL and 10 µg/mL Aurodox was added to the other Falcon tubes, respectively. All tubes were incubated at 37 °C with shaking for 5 h, after which the OD₆₀₀ value was measured. All tubes were centrifuged to separate cell mass at 4000 *xg* for 15 min, and the supernatant was transferred to fresh tubes and filtered through the 0.2 µm syringe filters (Thermofisher). The filtered supernatant was precipitated with trichloroacetic acid (TCA) at a final concentration of 5% TCA while on ice for 30 min. The precipitated proteins were harvested as described above and washed with 500 µL of 100% acetone. A final 5-minute centrifugation step (4000 *xg*) was performed to separate the acetone. The precipitated protein pellet was dried on a thermoblock for 5 min with the cap open to allow the acetone to evaporate. Twenty µL of 50 mM NaOH and 180 µL 1x SDS-PAGE loading buffer were added, the samples were boiled for 5 min at 95 °C, and the results were analysed using a Western blot (see section 2.8.3.)

2.10. Reverse transcription-quantitative PCR (RT-qPCR)

RT-qPCR was used to investigate whether the effect of Aurodox on EspC secretion is happening on a transcriptional level. Three biological replicates of EPEC E2348/69 treated with 0 µg/mL, 2.5 µg/mL and 5 µg/mL Aurodox were grown as described in 2.9. Following the 5-hour

incubation, the samples were stabilised to preserve RNA integrity. Briefly, 400 µL of the sample was added to 800 µL RNeasy Protect Bacteria Reagent (Qiagen) and vortexed immediately for 5 sec. The samples were incubated for a further 5 min at room temperature. The samples were centrifuged at 5000 $\times g$ for 10 min before discarding the supernatant and storing the samples at -80 °C for use in downstream RNA extractions.

2.10.1. RNA extraction

RNA extractions were performed using RNeasy® Protect Bacteria Mini Kit (Qiagen) according to manufacturer instructions (Protocols 4 and 7). Filter tips were used to protect from RNases, and all equipment and gloves were regularly treated with RNase AWAY (Fisher Scientific). RNA was eluted in 50 µL UltraPure™ Distilled Water (Invitrogen) and stored at -20 °C short-term or -80 °C long-term. RNA concentrations were measured using Nanodrop® ND-1000 (Nanodrop Technologies). TURBO DNA-free™ kit (Invitrogen) was used to digest DNA traces in the RNA samples according to manufacturer instructions. The quality of extracted RNA was evaluated using Agilent 2100 Bioanalyzer and Agilent RNA 6000 Nano Kit according to manufacturer instructions.

2.10.2. Reverse transcription for the synthesis of cDNA

LunaScript® RT SuperMix Kit (NEB®) was used to assemble reverse transcription (RT) reactions for RNA samples and no-RT reactions (negative controls) according to the manufacturer's instructions. Following cDNA synthesis, they were stored at -20 °C.

2.10.3. RT-qPCR and result validation

PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™) was used for all qPCR reactions and controls, and the total qPCR volume was 20 µL. Using its in-built software, RT-qPCR was performed in 7500 Real-

Time PCR Systems (Applied Biosystems™). MicroAmp® Optical 96-Well Reaction Plate and Optical Adhesive Film kits were used for sample loading (Applied Biosystems™). Genomic DNA (gDNA) of EPEC E2348/69 was extracted using GenElute™ Bacterial Genomic DNA Kit (Sigma) according to manufacturer instructions. The concentration of gDNA was quantified using Nanodrop® ND-1000 (Nanodrop Technologies) before making four serial dilutions of it (dilution factor 1/10). All samples were tested in three technical replicates. Primers that were already published for use in RT-qPCR were used to amplify *espC* (Table 3, (219)) and 16S ribosomal RNA (*rrnB*) - the endogenous control (Table 3, (220)). Primer efficiency (final concentration 300 nM) was tested using serial dilutions of EPEC E2348/69 gDNA to create standard curves. Accordingly, serial dilutions of cDNA were also performed to test primer efficiency. Finally, the cDNA of all no-RT reactions from section 2.10.2. was tested for DNA contamination using rtMS3-4 primers (see Table 2.3.). A standard dissociation curve program was run with the following cycling conditions: i) hold – 95 °C, 10 min, ii) denature – 95 °C, 15 sec, iii) anneal – 60 °C, 30 min, repeat ii and iii for 40 cycles.

2.11. *In vivo* photo-crosslinking using pBpa unnatural amino acid

This method was adapted from Farrell and Toroney (222) and optimised to use with existing EspC constructs. The plasmid containing the tRNA synthetase/tRNA orthogonal pair (pULTRA-pBpaRS) for the *in vivo* incorporation of the photo-crosslinker pBpa (p-benzoyl-L-phenylalanine, Merck) was a gift from Peter Schultz (197). Plasmid DNA of the native EspC (pLA33C1), EspC_{TAG951} containing the amber stop codon (pLA33C1TAG₉₅₁) and pULTRA-pBpaRS was transformed into DH10B cells (recommended to us for dual transformation due to its high competency). The transformants were cultured overnight in 2x YT medium with appropriate antibiotics at 37 °C with shaking at 200 rpm. Overnights were normalised to OD₆₀₀ 0.05 and added to pre-warmed

flasks (1 hour at 37 °C) containing glycerol minimal media (see Table 2.11.). To flask #4, which underwent photo-crosslinking, pBpa was added to a final concentration of 1 mM. The flasks were grown at 37 °C, shaking until OD₆₀₀ 0.6-0.8 (~ 18-20 h), after which 0.02% arabinose was added to induce EspC/EspC_{TAG951} expression (and 1 mM IPTG for the induction of pULTRA-pBpaRS when needed), followed by incubation for another 20 h as before. The flasks were harvested in 50 mL aliquots at 5000 xg for 5 min, and their pellets were stored at -80 °C for further analysis.

Component	Volume	
1.25% glycerol	80 mL	autoclaved
5x M9 salts	20 mL	autoclaved
Leucine (4 mg/mL)	1 mL	autoclaved
CaCl ₂ x 2 H ₂ O (20 mg/mL)	100 µL	autoclaved
MgSO ₄ (0.12 g/mL)	100 µL	autoclaved
D-biotin (5 mg/mL)	20 µL	sterile-filtered
Thiamine-HCl (5 mg/mL)	20 µL	sterile-filtered

Table 2.12. Recipe for the minimal glycerol media used to express EspC with an amber codon (TAG) engineered at codon 951 of the *espC* *ori*.

Each pellet was resuspended in 12 mL 1x PBS, but only the #4 – Photo-crosslinking sample underwent UV irradiation. The #4 resuspension was divided into four Pyrex® Petri dishes (80 x 20 mm, P35364D2, Scientific Laboratory Supplies), each containing 3 mL of the resuspended pellet, labelled with irradiation times (UV₃₆₅ 0m, UV₃₆₅ 30m, UV₃₆₅ 60m, UV₃₆₅ 120m). A handheld UV lamp was turned on 15 min before use, positioned 7 cm above the sample level (not the petri dish lid) and not turned off for the duration of the experiment. The corresponding #4 samples were taken at their time points (0 min, 30 min, 60 min, 120 min) from the UV set-up and transferred to 4 °C until each petri dish was cooled down for 2 h, including the irradiation period. The samples were removed from the

dishes using 1 mL 1x PBS. One millilitre of each irradiated sample was set aside for SDS-PAGE analysis, along with 1 mL of control samples (resuspended pellets #1, #2 and #3, see Table 2.12.). The 1 mL samples were centrifuged and resuspended in 50 μ L 1x PBS, 50 μ L 2x SDS-PAGE loading buffer and sonicated for 15 sec at amplitude 5 to reduce viscosity. The lysates were boiled at 95 °C for 10 min and loaded onto NuPage 3-8% Tris-acetate commercial gels (Invitrogen) along with Spectra™ Multicolor High Range Protein Ladder (26625, Thermo Scientific™). The gel was run at 100 V in Tris-acetate running buffer (50 mM Tricine, 50 mM Tris Base, 0.1% SDS, pH 8.24) until there was good band resolution between higher range molecular markers (~2 h). The gels were used for Western blotting as described in section 2.8.3., while the unused 3 mL irradiated cultures underwent immunoprecipitation (see section 2.17.)

Trial	#1 Native protein	#2 Background UAG	#3 No pBpa control	#4 Photo- crosslinking
expression vector	pLA33C1	pLA33C1- TAG ₉₅₁	pLA33C1- TAG ₉₅₁	pLA33C1- TAG ₉₅₁
pULTRA- pBpaRS	x	x	✓	✓
1 mM pBpa	x	x	x	✓
antibiotics	40 μ g/mL Cm	40 μ g/mL Cm	40 μ g/mL Cm, 50 μ g/mL Sm	40 μ g/mL Cm, 50 μ g/mL Sm

Table 2.13. Table outlining the constructs and growth conditions used to perform pBpa photo-crosslinking. The method was adapted from Farrell and Toroney (222).

2.12. FIAsh-EDT₂ labelling of tetracysteine-tagged recombinant proteins

The TC-FIAsh™ II In-Cell Tetracysteine Tag Detection Kit (Invitrogen™) was used to bind to pLA33mreB-TC according to the manufacturer instructions. Briefly, overnight cultures were diluted to OD₆₀₀ of 0.05. Recombinant TC-tagged protein expression was induced with 0.02% arabinose and incubated for 3 h (~ 0.6 OD₆₀₀). 1x N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES buffer, Sigma) was prepared in dH₂O, autoclaved, filtered through 0.2 µL syringe filters (ThermoFisher) and chilled at 4 °C. Following incubation, the cells were centrifuged at 4100 xg for 2 min and washed with ice-cold, 1x HEPES buffer. The pellet was resuspended in 1 mL to OD₆₀₀ of 1.0 using the same buffer. The centrifugation step was repeated, and the cells were resuspended in 500 µL 1x HEPES buffer containing 5 µM FIAsh-EDT₂ substrate and incubated for 45 min in darkness at room temperature. Subsequently, the cells were harvested as described before and washed twice with 1 mL 1x BAL buffer (British Anti-Lewisite 2,3 Dimercapto-1-propanol) wash buffer at the final concentration of 0.25 mM. The last wash with 1x HEPES buffer was performed as described before. The pellet was resuspended in 500 µL of the same buffer. The cells were mounted on slides and examined under the fluorescence microscope (see sections 2.11. and 2.12.).

2.13. MreB disruption with MreB polymerisation inhibitor A22

E. coli MG1655 containing EspC-mCherry (pLA18C1mc) and MreB-TC (pLA33mreB-TC) was grown overnight in LB containing appropriate antibiotics. One millilitre of culture was added to 99 mL of LB broth containing antibiotics and 0.02% arabinose. The culture was incubated for 1 h at 37 °C with shaking (200 rpm), after which 10 µg/mL S-83,4-dichlorobenzene-isothiourea (A22) was added to prevent MreB polymerisation. The cells were incubated for 3 h, harvested and prepared for microscopy as described in sections 2.9., 2.11. and 2.12.

2.14. Fluorescence microscopy

To check cell morphology and fluorescence signal before SR-SIM, a Nikon Eclipse 50i fluorescence microscope was used and fitted with a Nikon DS FI1 camera, Nikon Digital Sight Camera Control Unit DS-U3 and the Nikon Intensilight C-HGFI Precentered Fiber Illuminator. Microscope glass slides were prepared with a thin layer of 1.2% agarose, and 1 μ L of bacterial cell culture was mounted per sample, air-dried and covered with a coverslip. The NIS Elements software was used to capture images of samples observed under the Nikon Plan Fluor 100x/1.39 Oil objective. The images were processed using ImageJ software using the JaCoP toolkit for colocalisation analyses (223).

2.15. Super-resolution Structured Illumination Microscopy

The Zeiss Elyra PS.1 Microscope was used for all super-resolution imaging of EspC-mCherry and MreB-TC. Different objectives, mounting and fixation methods were used to enhance the fluorescence signal and optimise the samples for 3D imaging. Either C-Apochromat 63x/1.2W Korr M27 water immersion or Apo 63x/1.4 oil immersion objectives were used in Structured Illumination Microscopy (SIM) mode (488 nm MreB-TC 8-16%, 561 nm mCherry 8-16%). Beam splitters settings were BP 570-620 + LP 750 for mCherry and BP495-550 + LP 750 for TC-tagged proteins.

2.15.1. Mounting methods

The most common method used for mounting samples was live agarose-pad mounting. 1.2% agarose was prepared and poured into a petri dish to solidify (~7 mm thickness). A scalpel was used to excise a small cube of solidified agarose, and it was turned over to provide a smooth surface for mounting. Four μ L induced bacterial culture was mounted onto the agar, along with 1 μ L diluted TetraSpeck™ microspheres (T7279, Invitrogen) used to calibrate microscope settings (e.g. correct image

distortion/elongation to increase the validity of 3D imaging). The pad was left to dry, then placed on a MatTek glass dish (35 mm, no. 1.5) facing down and examined under the microscope. For imaging optimisation, several other methods were used (addressed in the results as needed). Samples mounted with CitiFluor CFM3 high refractive index glycerol-based mountant were placed on Fisherbrand (1157-2203) microscope slides using Zeiss cover glasses (no. 1.5, high-performance, $0,170 \pm 0,005$ mm) and CoverGrip™ Coverslip Sealant (Biotium).

2.15.2. Cell fixation

Cells expressing EspC-mCherry (see section 2.4.) were fixed by treatment with 0.1% glutaraldehyde (GA) for 10 min and stored for up to a week at 4 °C. Aliquots (1 mL 0.1% GA) were prepared by diluting the 25% GA (G5882 Sigma) in sterile dH₂O and storing at -20°C until needed. When indicated, poly-D-Lysine adhesive was used to adhere cells to the coverslips. Coverslips were treated with 150 µL poly-D-lysine (Gibco™) for 15 min before washing them with sterile dH₂O and mounting the samples.

2.16. Figure preparation

Unless otherwise specified, figures were prepared using BioRender Premium. Tables were made in Microsoft Word, while GraphPad was used to create graphs and statistical analyses.

3. Validation of EspC-mCherry and EspC-TC constructs to study protein localisation in *E. coli* MG1655

3.1. Introduction

While fluorescent labelling of surface-exposed proteins can be achieved with non-invasive techniques, proteins also need to be imaged within the confines of the bacterial cell to track their translocation pathways and partners. Genetically encoded FPs are widely used tags for *in vivo* imaging. Once expressed and folded into their intrinsically-fluorescent structures, they fluoresce without relying on external factors (except oxygen availability). The Green Fluorescent Protein (GFP) is the most commonly used FP, but its green emission can overlap with autofluorescence, reducing the signal-noise ratio in many bacterial species. To study bacterial protein-protein interactions (PPIs), fluorophores with a broader range of fluorescence spectra were needed to minimise signal interference. Red-shifted FPs provide more photostability and cause less light scattering, which is essential for super-resolution microscopy. The problems with tagging with polymers (i.e. proteins) are their size and propensity to self-aggregate (cluster), so they can impair native protein functionality. mCherry is a synthetically derived red FP, approximately the same size as GFP on average (~27 kDa) (224). It is attractive for imaging because it is a true monomer and can fluoresce in the oxidising environment of the periplasm (225, 226, 227). However, mCherry clustering artefacts have been reported when fused to the small Clp protease (~43 kDa) (228).

Hybrid tags have an advantage over FPs because they combine genetically encoded small peptide tags with highly specific, easily washable fluorescent dyes. The oldest of those systems still in use today is FIAsh-EDT₂ labelling (**F**luorescein **A**rsenical **H**airpin binder – ethanedithiol) which uses biarsenicals fused to fluorescein to bind the tetracysteine (TC) motif with high specificity and affinity (CCXXCC, where

X denotes any non-cysteine aa) (229). The positioning of the biarsenicals within fluorescein results in high specificity for grouped cysteines, which FIAsh (<700 Da) prefers to bind over EDT₂. Non-specific labelling is minimised by using BAL (British Anti Lewisite) buffer, which outcompetes FIAsh binding to thiols of native cysteines. Excessive washing also leads to FIAsh being outcompeted for the TC motifs (229). The thiol groups of the four cysteines need to be reduced to bind the TC motif, limiting FIAsh to the cytoplasm unless the cysteines are acutely reduced (e.g. using tributylphosphine) (230, 231).

It is necessary to confirm if the tagged protein can translocate across the membranes when it comes to imaging secreted virulence factors like ATs, especially those released into the extracellular milieu following OM translocation (e.g. EspC) or if their observed subcellular localisation is a result of clustering. SpyTag-SpyCatcher (STSC) relies upon an interaction between the polypeptide SpyTag (ST, 39 nucleotides) and its partner protein SpyCatcher-sfGFP at the OM (see section 1.8.3, Fig. 3.1.)

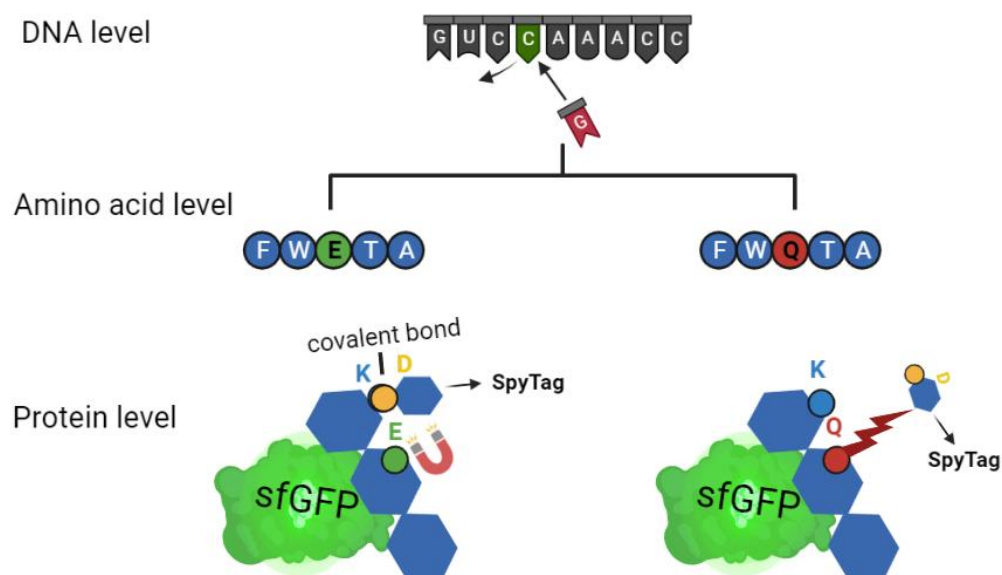


Figure 3.1. SpyCatcher-sfGFP is available in active (E78) and inactive (Q78) form. SpyCatcher-sfGFP (SC) binds the SpyTag (ST) only when there is a glutamate at residue 78. ST-SC binding happens between SC lysine (K) and ST aspartate (D), facilitated by a neighbouring SC glutamate (E). By substituting guanine (232nd base in SpyCatcher ORF) for cytosine at the DNA level (top), E in SC becomes glutamine (Q) (middle). Q is unable to facilitate ST-SC binding and is therefore used as a negative control for background fluorescence (bottom right). The magnet represents an attractive force facilitating ST-SC binding, while the red spark represents ST bouncing back from SC-EQ.

(193). Using this technology, the YrInv TAA from *Yersinia ruckeri* was recently visualised on the cell surface, but STSC can also be used to quantify OM translocation (195, 232).

Although the effect of ATs on mammalian cells has been an active area of investigation, the studies were not designed to understand AT biogenesis. Due to their elegant secretion system, surface-tethered ATs have been of interest for the development of new vaccines using antigen autodisplay since the late '90s (233). By substituting the passenger for known antigens, non-virulent strains with surface-exposed passenger antigens could be a safe and effective way of developing specific immunity (234, 235). ATs tested for their effects on host cells mainly included surface-tethered ATs (236, 237), or the host cells were the ones tagged (238, 239, 240, 241, 242). Polar localisation of several classical ATs has been reported in the past for surface-tethered IcsA and AIDA-I, but also for a SPATE SepA cleaved off when it reaches the OM, leading to the argument that T5 'machinery' is situated at the cell poles (109, 187). Surprisingly, Ashawesh *et al.* (2018) found two other classical ATs, EspC and AaaA, to localise into discrete patches along the cell using mCherry and FIAsh-EDT₂ tags (206).

This previous study did not fully quantitate the observed EspC localisation. It thus remained possible that the large size of mCherry could have affected EspC folding and secretion. Consequently, although Ashawesh (206, 207) detected EspC-mCherry fusions in culture supernatants of *E. coli* MG1655, the possibility of intracellular protein leakage could not be ruled out. To tackle this issue, an alternative method for tracking EspC secretion was needed to enable the discrimination between intracellular intermediates and extracellular EspC. Because of the size of SC-sfGFP, it can only bind ST proteins that have successfully translocated across the OM (see section 1.8.3.). By engineering ST into EspC and EspC-mCherry, it would be possible to quantify and compare the OM secretion efficiency of these plasmid-encoded proteins by quantifying OM fluorescence in a plate reader. It was hypothesized that the amounts of EspC-mCherry-ST and native EspC-ST would not be

significantly different if both could reach the bacterium's outer surface with similar efficiency. Detection of EspC-mCherry-ST on the surface of intact bacteria would strengthen the argument that the patchy, helical localisation patterns observed for EspC-mCherry are bonafide intermediates in the T5SS pathway.

The EspC-ST would have additional utility. For example, it could be produced in mutants of *E. coli* predicted to have deficient T5SS. For example, strains containing temperature-sensitive (ts) Sec translocon mutations. Finally, due to its small size, EspC-ST could be used to explore its translocation into host cells, which utilises the T3SS (73, 243).

3.2. Results

Commonly used FPs have their drawbacks, being bulky and prone to homodimerisation. Even true monomers have now been shown to be potentially disruptive to normal protein function by causing clustering artefacts that can be mistaken for a particular subcellular location, even though it consistently remains a monomer in FP-aggregation quantification studies (227, 228). Having identified the STSC approach as suitable to fluorescently label extracellular ATs because the SpyCatcher substrate could not enter the cell, the tools required were engineered.

Dr Jack Leo (NTU) kindly provided the plasmids encoding SpyCatcher-sfGFP and its inactive form SpyCatcherEQ-sfGFP (Table 2.2.). The substitution of E-Q at residue 78 was verified by Sanger sequencing. Ashawesh's pBAD EspC and EspC-mCherry constructs were used for ST-SC labelling (Table 2.2.) (206). Ashawesh cloned these fusion proteins into two vectors, pBAD18 (higher copy number, Amp^R) and pBAD33 (medium copy number, Cm^R), with both used in EspC localisation experiments (pBAD33 constructs) (206, 207). This study uses them for EspC-MreB colocalisation experiments (MreB-TC is only available in the pBAD33 vector).

Tagging mCherry with TC was attempted to serve as a localisation control (native mCherry distributes diffusely in the cytoplasm under native conditions). By visualising it before and after FIAsh-EDT₂ labelling, it would be possible to see if FIAsh-EDT₂ labelling affects cell morphology and protein localisation, and confirm that the substrate can enter the bacterial cell to interact with the TC tag. Additionally, on a technical level, visualising mCherry-TC in the green channel only after FIAsh-EDT₂ labelling would eliminate the possibility that the red channel (mCherry) causes fluorescent bleedthrough to the green channel.

3.2.1. Purification of SpyCatcher-sfGFP fusions

To have the fluorescent SC substrate available and validated, ready for coupling to genetically engineered STs in the proteins chosen for tagging, the first step was to produce and purify the SC-sfGFP. The conventional affinity chromatography was facilitated by the visual tracking of the green fluorescence of sfGFP (superfolder Green Fluorescent Protein), enabling the identification of eluted SpyCatcher in fractions for collection. The efficiency and purity of Ni-IMAC were verified on SDS-PAGE, and the elution chromatogram was used to pick fractions for downstream analysis (Fig. 3.2., 3.3.).

Aliquots were taken during Ni-IMAC, as listed in Table 2.10. Following cell lysis, aliquots of the cell pellet (P) and the cell lysate (Ls) were taken to test lysis efficiency. Next, as the lysate flowed through the Ni-IMAC column, the flowthrough (F) was collected to show the column specificity. A wash (W) aliquot was also taken to ensure no SpyCatcher-sfGFP was dislodged from the column following the wash. The protein was eluted using Äkta Pure to obtain chromatograms and determine the appropriate concentration of the elution buffer needed to elute the two sfGFP fusions (Fig. 3.2., 3.3.). The chromatograms in Figure 3.2. and 3.3. show which fractions (x-axis) contain eluted proteins (blue peaks) which are detected by UV (y-axis). The green diagonal line indicates the concentration gradient of the elution buffer. The orange diagonal monitored pre-column pressure to avoid damaging the column during elution.

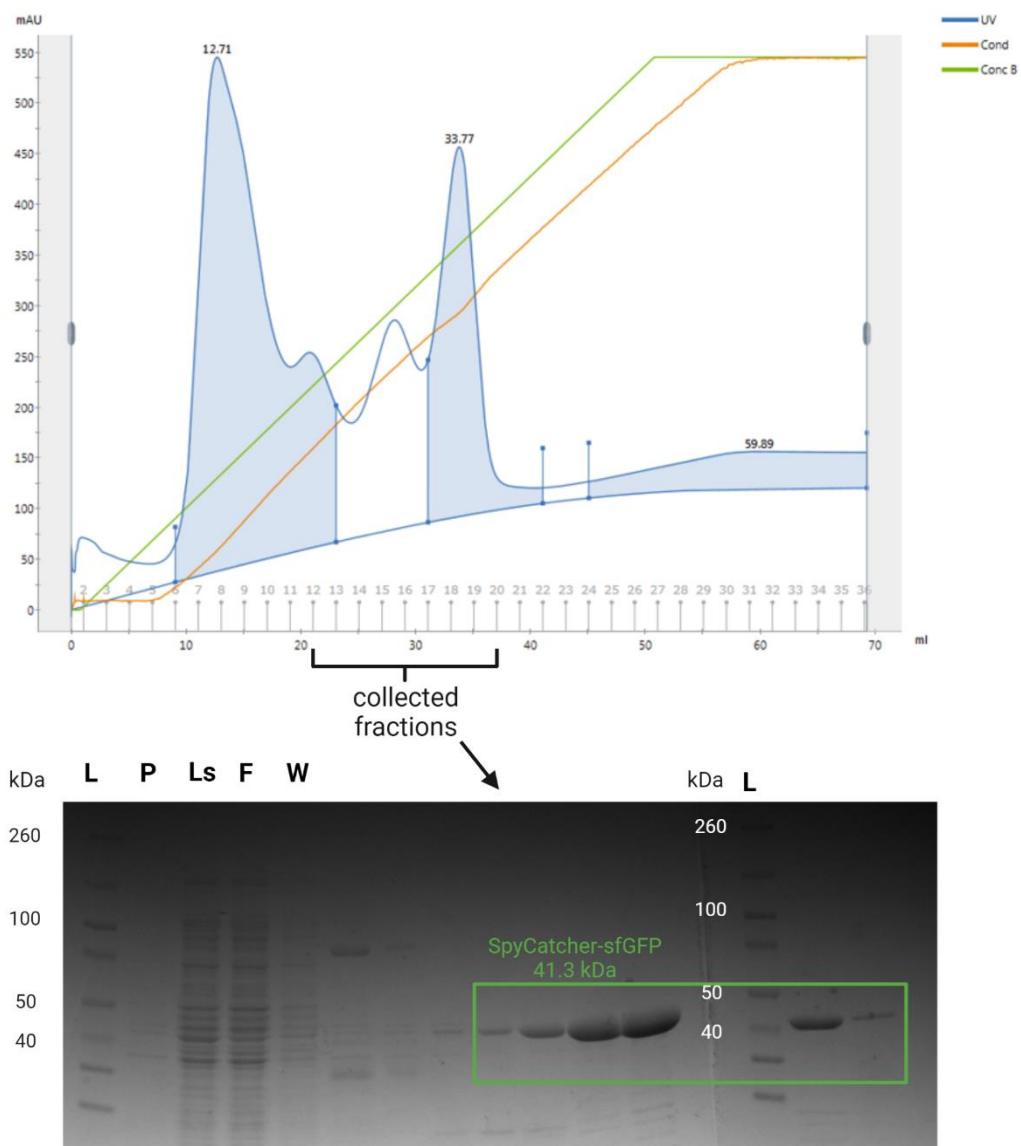


Figure 3.2. SpyCatcher-sfGFP was successfully purified using Ni-IMAC. The chromatogram recorded peaks containing large amounts of protein and visual inspection (green colour of fractions) confirmed SpyCatcher-sfGFP was in fractions 17-19 (see text). In total, fractions 12-20 were picked and SDS-PAGE was run as described in 2.8.2. with controls (see text). Fractions 15-20 were chosen for protein concentration (green rectangle). L – ladder, P – lysis pellet, Ls – lysate, F – flowthrough, W – wash. Molecular weights of marker proteins are indicated in kDa, and the green box highlights the SC-sfGFP.

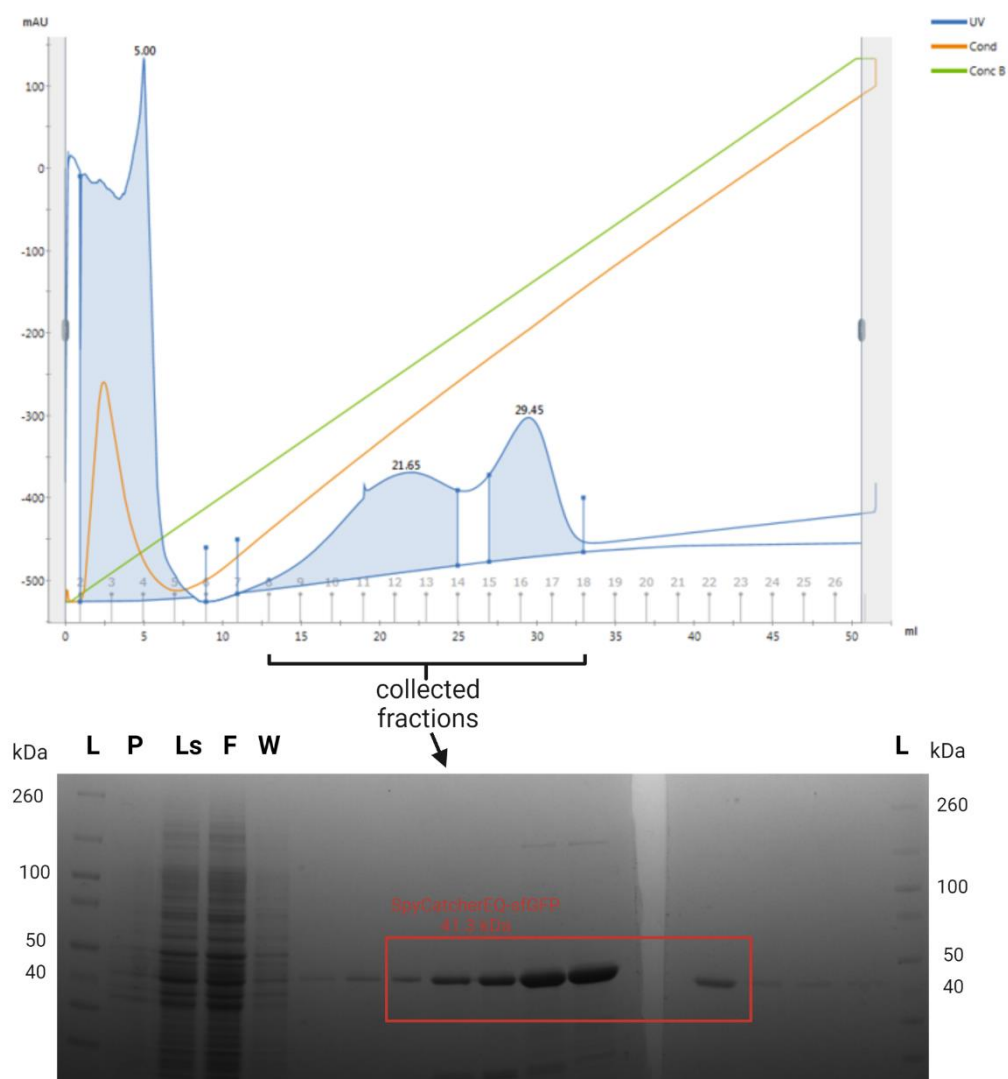


Figure 3.3. SpyCatcherEQ-sfGFP was successfully purified using Ni-IMAC. The chromatogram recorded peaks containing large amounts of protein and visual inspection (green colour of fractions) confirmed SpyCatcher-sfGFP was in fractions 12-17 (see text). In total, fractions 8-18 were picked and SDS-PAGE was run as described in 2.8.2. with controls (see text). Fractions 10-15 were chosen for protein concentration (red rectangle). L – ladder, P – lysis pellet, Ls – lysate, F – flowthrough, W - wash. Molecular weights of marker proteins are indicated in kDa, and the green box highlights the SC-sfGFP.

3.2.2. Introducing SpyTag in the middle of the EspC passenger

The SpyTag insertion site was chosen to be between S349 (serine at position 349 of *espC* open reading frame [ORF]) and I350 (isoleucine at position 350 of *espC* ORF) or S588 and I589 of the *espC-mCherry* ORF (Fig 3.4.).

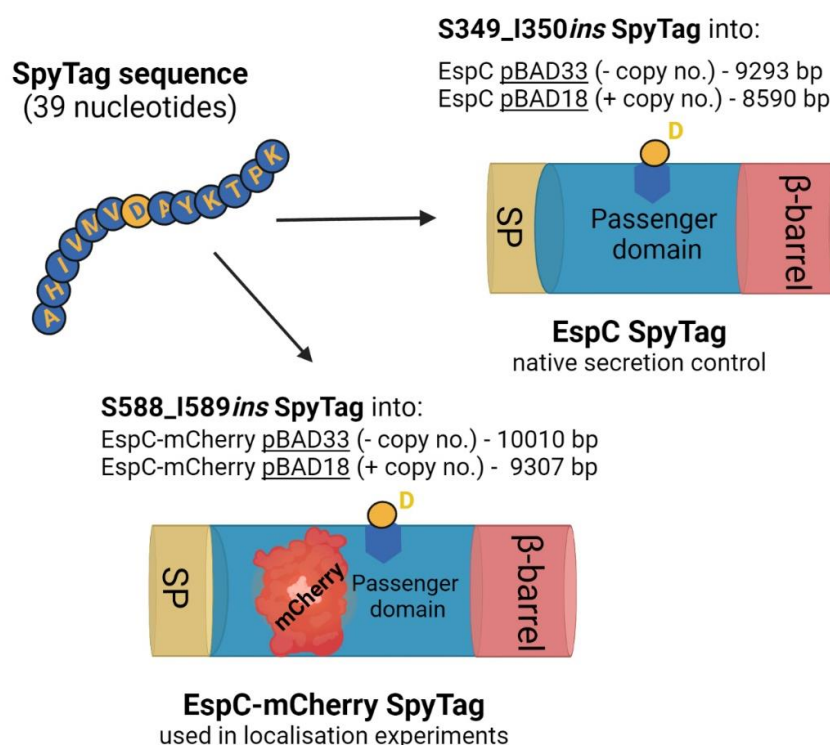


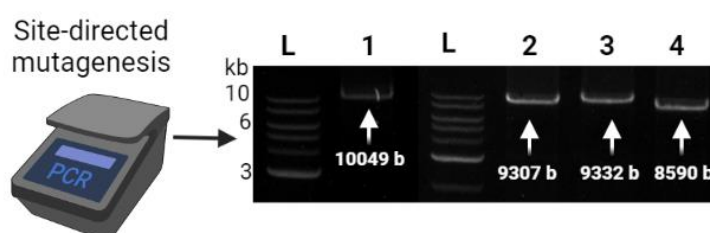
Figure 3.4. Cloning strategy for the incorporation of the SpyTag polypeptide into EspC and EspC-mCherry. The site for the incorporation of SpyTag was chosen to be immediately downstream of the site where mCherry was successfully inserted in the past. It was hypothesised a small mutation at this site would not influence protein folding

Closely upstream of this site was where mCherry was previously successfully inserted by my predecessors, with hopes it would be safe to insert 13 more residues there without disrupting protein folding (207). Because of the potential of plasmid compatibility issues arising in future experiments and the likelihood of long-term use of EspC-ST, ST (AHIVMVDPYKTPK) was engineered into four EspC constructs from two different vectors (pBAD33 and pBAD18) to create low and high copy number versions with different antibiotic selections. SDM was used to engineer SpyTag into native EspC (pLA33C1, pLADC1) and EspC-

mCherry (pLA33C1mc, pLA18c1mc) to compare efficacies translocating across the OM by using native EspC constructs as positive controls. SDM primers (MS12-13) and their ideal annealing temperatures (T_a) were determined using NEBasechanger® (Table 2.3.).

SDM was performed using Q5® Site-Directed Mutagenesis Kit (E0554, NEB) due to its low error rate observed while performing SDM throughout this study (Fig. 3.5.A.). Thermocycling conditions are listed in Table 2.4. The PCR products were processed with the provided Kinase-Ligase-DpnI (KLD) mix to remove template DNA and circularise the product (before chemically transforming them in the provided DH5α competent *E. coli* E0554, NEB). After a few failed attempts at transformation, a switch to T4 polynucleotide kinase (PNK) and T4 ligase was made, with the method described in Table 2.6. Before switching, a final effort using the KLD mix was performed by adding a dialysis step to facilitate the

A



B



Figure 3.5. The PCR step of site-directed mutagenesis was successful. SpyTag insertion confirmed in control EspC constructs. SDM PCR products were analysed on 1% agarose gels as described in 2.6. The 1 kb DNA ladder (L) was used to verify the sizes of EspC-mCherry-ST pLA33c1mcST (1) & pLA18c1mcST (2), and native EspC pLA33C1ST (3) & pLA18c1ST (4) (A). Both native EspC constructs were sequenced to show successful SpyTag integration between S349 and I350 (B).

exchange of the high-salt KLD Reaction Buffer for dH₂O. MF-Millipore™ 0.025 µm MCE 13 mm membranes (Merck) were placed in a petri dish containing sterile dH₂O, and 5 µL of the KLD-processed products were pipetted onto the membranes and incubated at room temperature for 45 min. All of the product was pipetted and used in transformations, but the transformations were still unsuccessful. Following the switch to T4 PNK and ligase, transformants were obtained. The insertion of SpyTag in the native EspC in both pBAD33 and pBAD18 vectors was verified by sequencing, and an STSC binding assay was performed, as described in section 2.8. (Fig. 3.5.B., Table 3.1.).

	#1	#2	
EspC	28798	29501	RQ
EspC-STa	47730	47439	
EspC-STb	48169	48711	

Table 3.1. EspC-STa (pLA33C1ST1) and EspC-STb (pLA18c1ST1) successfully bind SpyCatcher-sfGFP after OM translocation. The table shows the relative quantification (RQ) of fluorescence EspC-STab (with ST inserted between S349 and I350) and SpyCatcher-sfGFP binding at the OM. The RQ observed for EspC-STa, and EspC-STb is distinctively higher than the background fluorescence in the negative control (EspC). The method is described in 2.8. Briefly, following recombinant protein induction, the cells were harvested, normalised to 1.0 OD₆₀₀ and incubated with 10 µL 10 mg/mL SpyCatcher-sfGFP for 60 min. After three PBS washes, the cells were resuspended in 200 µL PBS. A plate reader was used to measure fluorescence (absorbance – 485 nm, emission 535 nm) on a black 96-well polystyrene plate. RQ – relative quantification.

Although multiple transformants of EspC-mCherry-ST were obtained, sequencing results showed that SpyTag wasn't successfully integrated into either of the constructs (pLA33C1mc, pLA18c1mc) (Fig. 3.6). All pLA33C1mcST transformants displayed noticeably slower growth (data not shown) and were unable to be sequenced at the desired region (Fig. 3.6.).

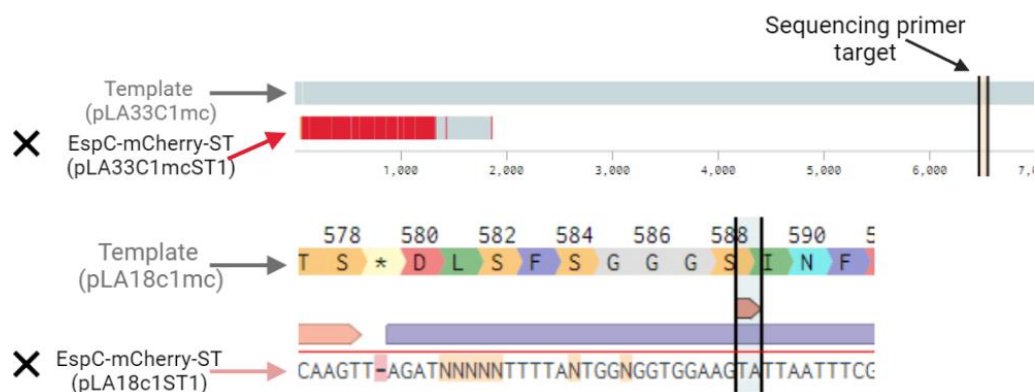


Figure 3.6. The SpyTag sequence was not successfully inserted into either of the EspC-mCherry constructs. After obtaining multiple transformants of EspC-mCherry-ST from both vectors, sequencing revealed pLA33C1mcST1 colonies were aberrant with the sequencing primer hybridising to the wrong place. Since the site is the same in all constructs and no hybridisation problems occurred elsewhere, these transformants were not analysed further.

3.2.3. Placing the ST tag closer to the N-terminus of the EspC passenger

It was decided to move the ST insertion site closer to the N-terminus. The new site was positioned directly after the SP cleavage site at positions Q55 and L56 (glutamine and leucine, respectively, Fig. 3.7.A). New primers (MS14, MS15) were designed, and the cloning was undertaken with the assistance of MSc student Glory Fernandes (Table 2.3). It was not clear if the PCR was successful, and unpurified PCR products were visualised in the wells of a 1% agarose gel, as described in section 2.5.2. (Fig. 3.7.B). Still, due to the intensity of the DNA bands, it was decided to still transform. The rest of the PCR product was treated with T4 PNK and ligase as described in section 2.6.2., but the transformations were unsuccessful. A modified version of the T4 treatment was performed where the PNK reaction was assembled using the T4 ligase buffer (as opposed to the PNK buffer); after heat inactivation, there was no clean-up step. Instead, 1 μ L T4 ligase and 2 μ L T4 ligase buffer were added to the heat-inactivated PNK reaction and incubated for 16 h at 16 °C. Before transforming the ligated PCR products, a dialysis step was added to facilitate buffer exchange for dH₂O, as described in section 3.2. The transformations were repeated using electrocompetent DH5 α and 5-alpha Competent *E. coli* (E0554, NEB) but were unsuccessful.

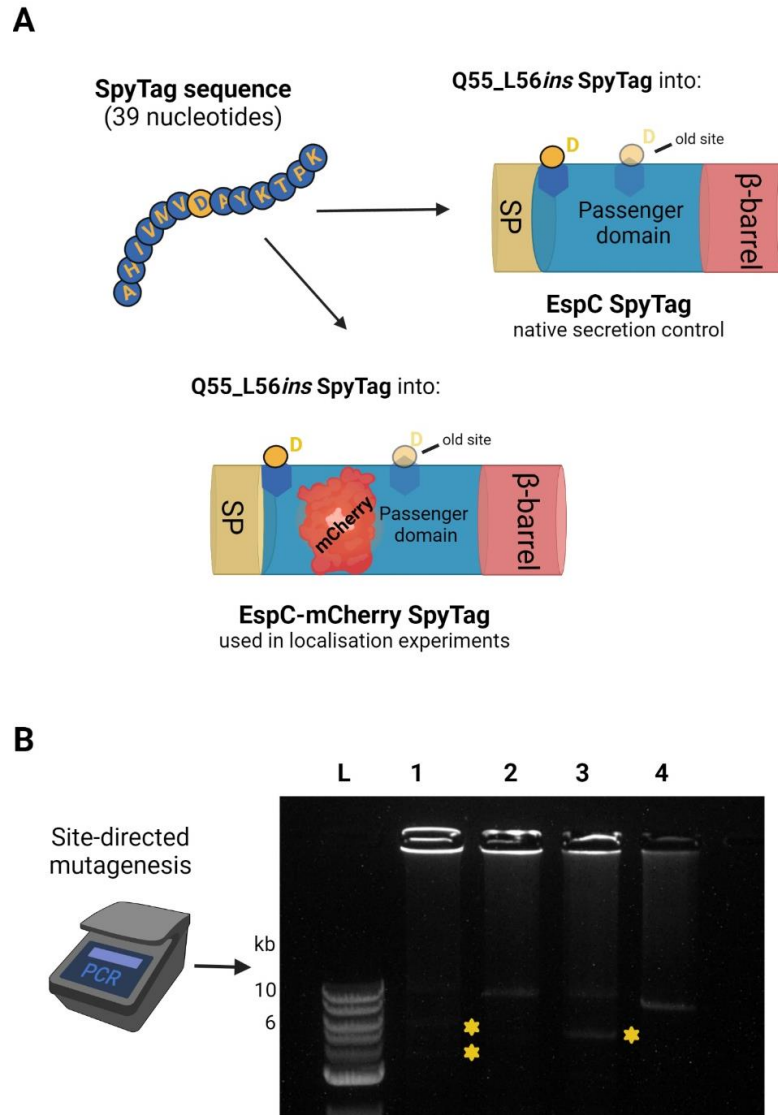


Figure 3.7. Cloning strategy for the insertion of SpyTag at the N-terminus of EspC and EspC-mCherry. The location of the new SpyTag insertion site is visualised in comparison to the old site (A). SDM using the MS14/15 primer pair was performed by Glory Fernandes as described in 2.6. Unpurified PCR products were visualised on 1% agarose gel. Samples in lanes 1-3 got stuck in their wells and their size couldn't be determined, although unlikely to be the required products, the intensity of the DNA was such that the subsequent transformation step was undertaken. The yellow asterisks denote contaminants and primer dimers which were later removed using the Wizard PCR Clean-up (Promega).

Although the crystal structure of EspC has not been solved yet, a predicted model was generated using AlphaFold, to help identify the ideal insertion site around the N-terminus of the EspC passenger (Fig. 3.8.) (244). A suitable 'loop' in the EspC structure was identified in a few residues upstream of the second site, between D59 and N60 (aspartate and asparagine, respectively).

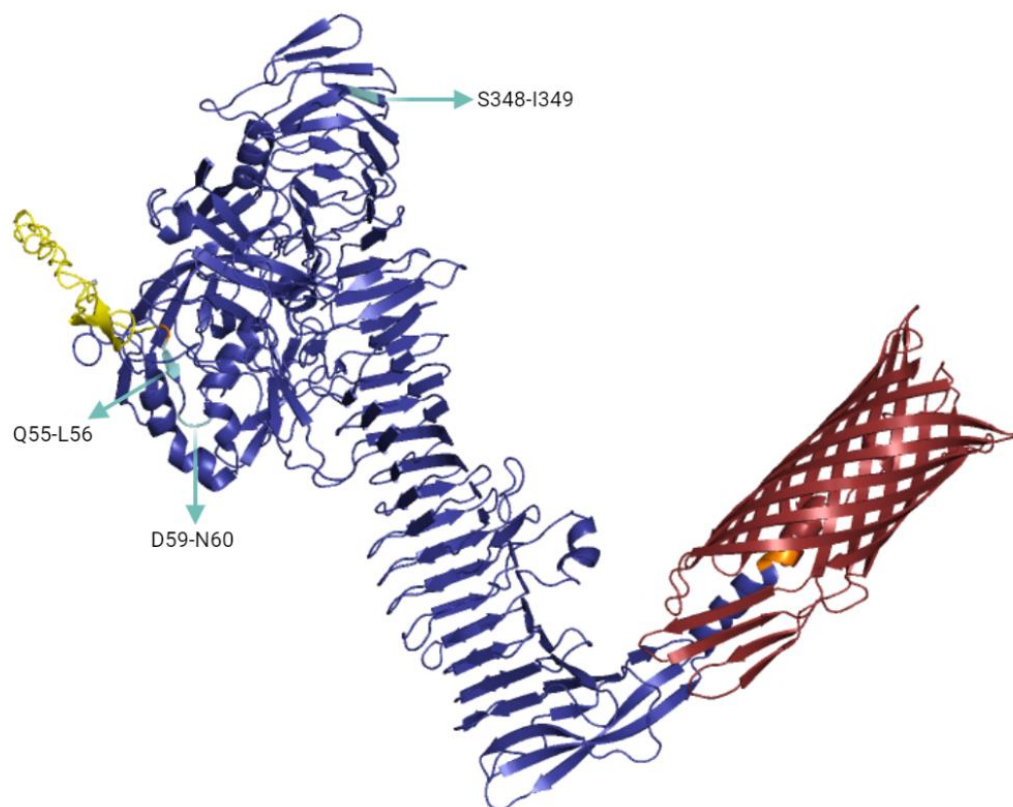


Figure 3.8. The predicted structure of EspC generated by AlphaFold. The light-blue arrows designate the first (S348-I349), the second (Q55-L56) and the third (D59-N60) site for attempted site-directed mutagenesis of SpyTag into EspC and EspC-mCherry. Signal peptide – yellow, passenger – dark blue, β -barrel – red, intramolecular cleavage sites - orange.

SDM was performed as described above, with a T_a gradient ranging from 50.6 °C to 71.4 °C. The ideal T_a of 62.4 °C (Fig. 3.9.A.) was chosen, and the modified T4 treatment was used to obtain colonies ($n = 16$) for all four EspC constructs and validate them through sequencing. Sequencing showed only the first few residues integrated successfully, with the rest of the sequence scrambled (Fig. 3.9.B.). Out of 16 colonies sent to sequencing, 15 were successfully sequenced with the MSSP primer (Table 2.3). A BLASTN search was run for the 13 residues showing up at the site of SpyTag insertion to see if primers were binding elsewhere in the genome non-specifically (245). Seven out of 15 sequences got positive hits for the EspC sequence. Their locations in the ORF are shown in Figure 3.10.

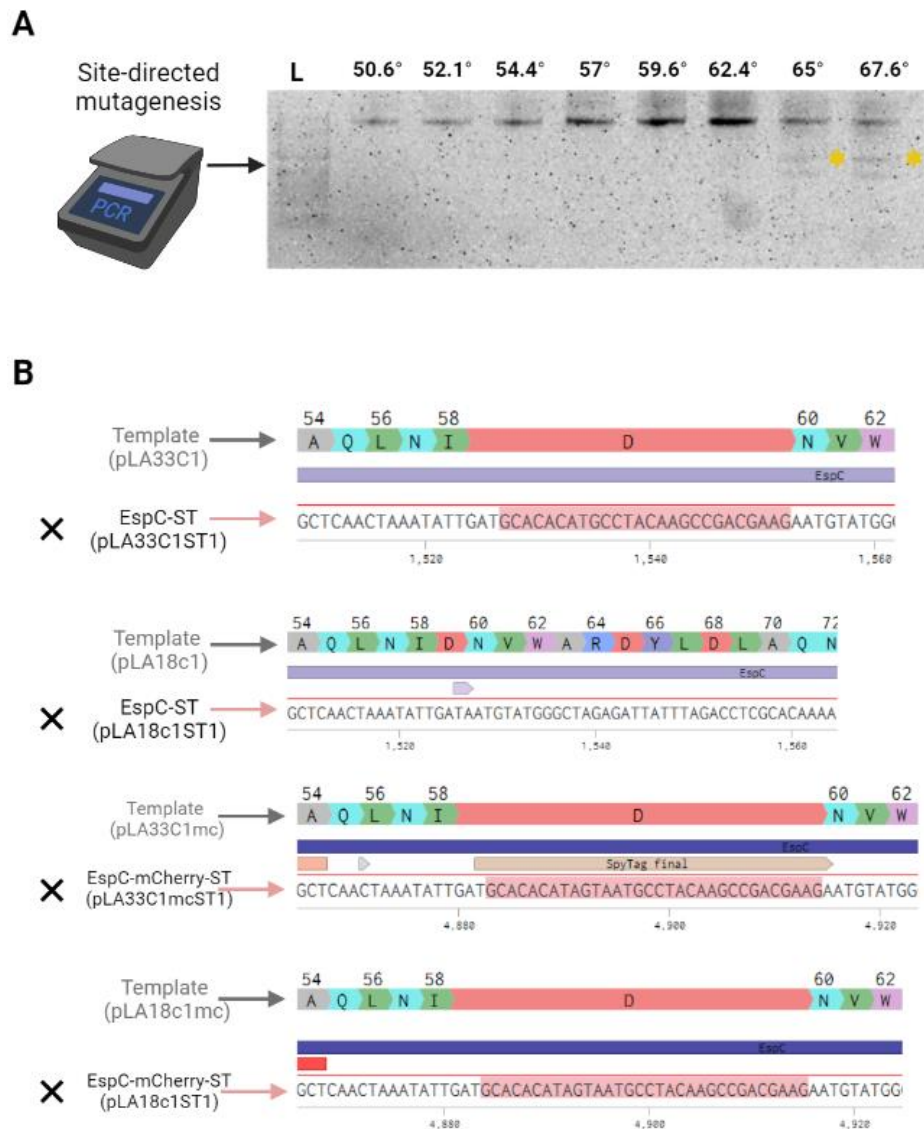


Figure 3.9. SpyTag insertion between D59 and N60 yielded many transformants but was ultimately unsuccessful. A representative electrophoresis gel of EspC PCR products generated by gradient PCR using the primer pair MS20/21. The ideal annealing temperature of 62.4° C was chosen for all four EspC(-mCherry) constructs. Some non-specific amplification occurred at higher annealing temperatures (yellow stars) (A). Representative sequence alignments show pLA18c1ST1 hadn't incorporated anything, while the other constructs all contain the same, non-specific sequence at the insertion site (B).

Insert: **GCA CAC ATA GTA ATG GTA GAC GCC TAC AAG CCG ACG AAG**

EspC-mCherry- ST pLA33C1mcST_{DN}:

1. **GCA CAC ATA GTA ATG** CCT ACA AGC CGA CGA AGA ATG TAT – no hits
2. **GCA CAC ATA GTA ATG** GTG GAG CTT ATA GTG TCA CAG CAA – no hits
3. **GCA CAC ATA GTA** AGC CTA CAA GCC GAC GAA GAA TGT ATG – no hits

EspC-mCherry-ST pLA18C1mcST_{DN}:

4. **GCA CAC ATA GTA** AGC CTA CAA GCC GAC GAA GAA TGT ATG – no hits
5. **GCA CAC ATA GTA** GCC TAC AAG CCG ACG AAG AAT GTA TGG – no hits
6. **GCA CAC ATG** CCT ACA AGC CGA CGA AGA ATG TAT GGG CTA – no hits
7. **GCA CAC ATA GTA** GCC TAC AAG CCG ACG AAG AAT GTA TGG – no hits

EspC-ST pLA33C1ST_{DN}:

8. **GCA CAC ATA TGT ATG** GGC TAG AGA TTA TTT AGA CCT CGC – EspC
9. **GCA CAC ATA TGT ATG** GGC TAG AGA TTA TTT AGA CCT CGC – EspC
10. **GCA CAC ATA GTA ATG** CCT ACA AGC CGA CGA AGA ATG TAT – no hits
11. **GCA CAC ATA GTA TGG** GCT AGA GAT TAT TTA GAC CTC GCA – EspC

EspC-ST pLADC1ST_{DN}:

12. **GCA ACC ATT AAT CTG GGA GAC GGT GAT GCT GAG ACG GAT** – EspC
13. **GCA CAC ATA GTA TGG** GCT AGA GAT TAT TTA GAC CTC GCA – EspC
14. **GCA CAA AAT AAG GGG GTG TTT AAA GCT GGT GCG ACC AAT** – EspC
15. **GCA CAC ATA GTA TGG** GCT AGA GAT TAT TTA GAC CTC GCA – EspC



Figure 3.10. Sequence analysis of the SpyTagged transformants following site-directed mutagenesis revealed non-specific binding of the SDM primers. Due to the fact colonies were easily obtained, there was interest in analysing the results to determine why SDM failed. A nucleotide BLAST search (default parameters) was run using 13 residue long sequences taken from the SpyTag insertion site D59-N60 of the transformants. Out of 15 in total, 7 sequences were 'hits'. Out of those 7, four different sequences were identified as belonging to the native *espC* gene. The plasmid pair MS20/21 bound non-specific downstream of the planned insertion site. Letters in bold indicate codons matching the SpyTag insert. Identical transformant sequences are highlighted in the same colour (left). The locations of EspC sequences obtained from transformants are shown in relation to the intended insertion site (right).

3.2.4. An attempt to introduce the SpyTag into EspC using DNA assembly

Following the repeated failure of inserting SpyTag (ST) into EspC-mCherry using SDM, it was decided the tag would be incorporated using DNA assembly (see section 2.5.3., Fig. 3.11.). Primer pairs 23-27 were designed using NEBuilder (NEB®) to amplify the SpyTag fragment from a successful ST mutant (pLA33C1ST1, Fig. 3.5.B.) and amplify EspC and EspC-mCherry (pLA33C1 and pLA33C1mc, respectively) fragments from position N60 to D59 of the *espC* ORF (Fig. 3.11-13). The fragments were ligated as described in section 2.5.6., along with a positive assembly control (NEB®) and the no-template negative control.

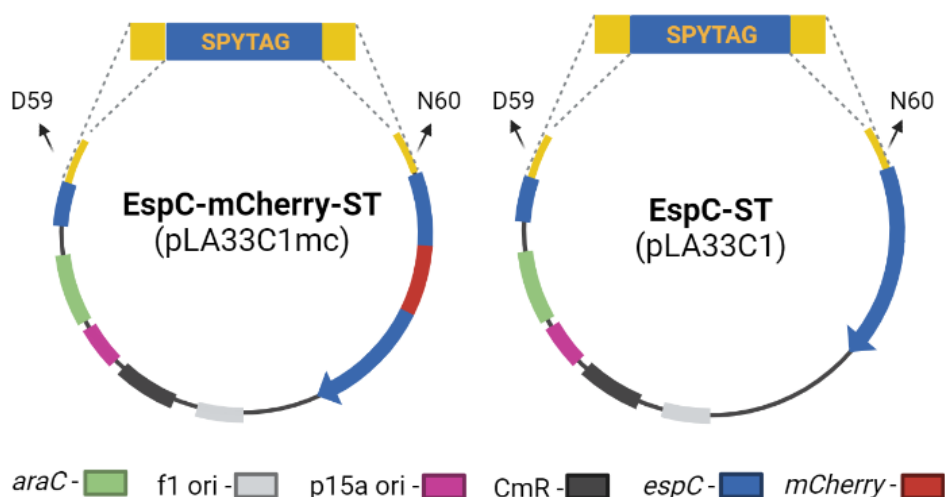


Figure 3.11. Strategy for the incorporation of SpyTag into EspC constructs using Hi-Fi DNA assembly. Primers were designed for the ligation of SpyTag to EspC pBAD33 constructs between residues D59 and N60. SpyTag was amplified from a previously SDM-generated mutant (pLA33C1, Fig. 3.5B) while the EspC and EspC-Cherry pBAD33 vectors were amplified from D59 to N60 position of *espC* ORF, respectively. The fragments were assembled in a 5:1 ratio (SpyTag : vector fragment), ligated at 50° C and transformed into Turbo competent *E. coli* (NEB®). For the full method, see section 2.5.6. ST – SpyTag; *araC* – L-arabinose operon; ori – origin of replication; CmR – chloramphenicol resistance cassette.

After ligation, 2 µL of all reactions were transformed into Turbo competent *E. coli* (NEB®) using chemical transformation, spread over LB agar plates and incubated overnight (see section 2.5.9.). Multiple colonies were obtained, and colony PCR was performed to test for the presence of the SpyTag fragment, but all obtained transformants were negative for SpyTag amplification (Fig. 3.12.A-B, Fig. 3.13.).

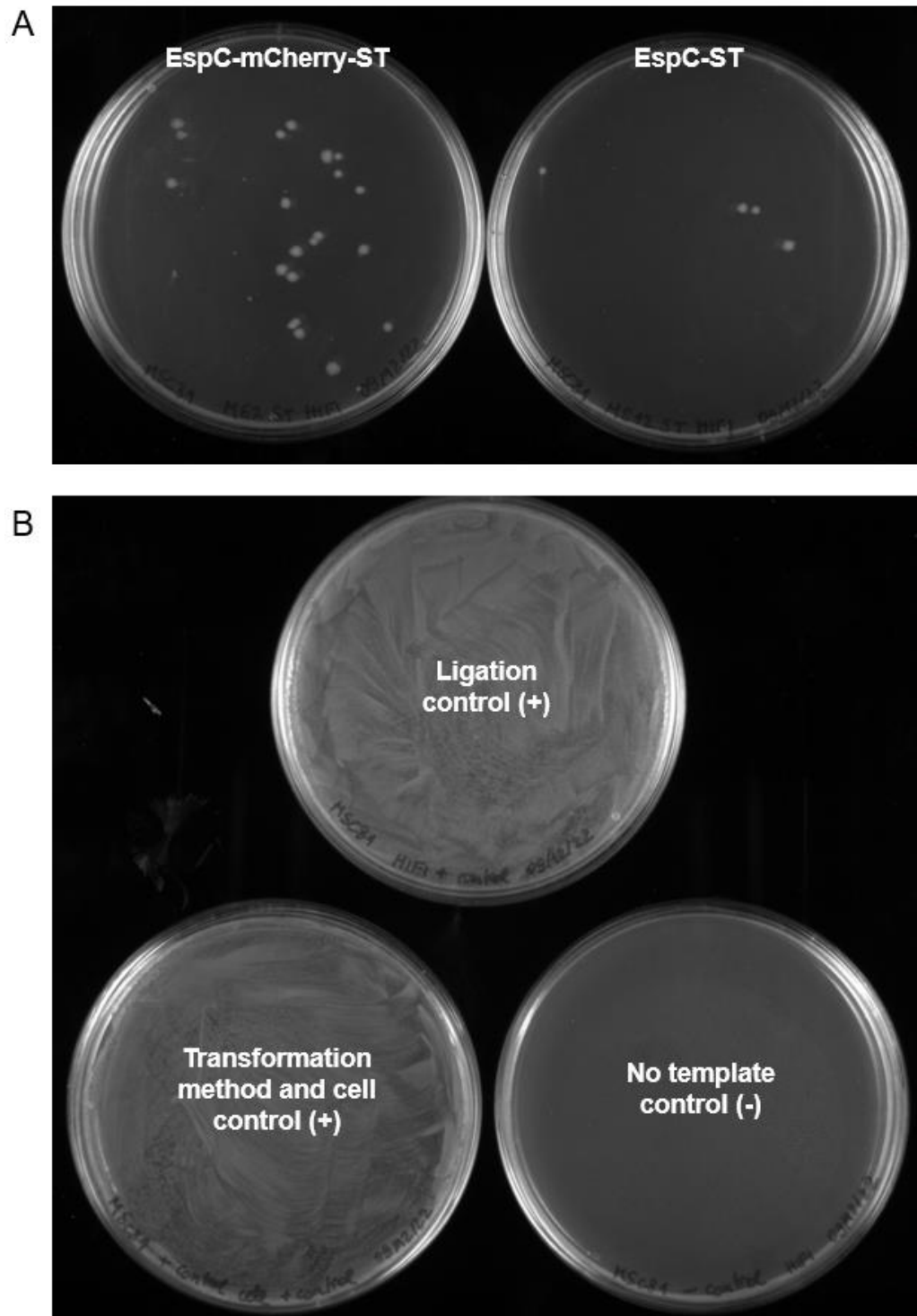


Figure 3.12. HiFi DNA Assembly was used to obtain EspC and EspC-mCherry mutants after unsuccessful SDM attempts. (A) Multiple colonies obtained following DNA assembly were picked for colony PCR. **(B)** EspC-mCherry (pBAD33c1mc) and EspC (pBAD33C1) were amplified from N60 to D59, creating ~9 kb DNA fragments. The ST DNA fragment was amplified from a EspC-ST mutant obtained previously (see Fig. 3.5.). The fragment ligation was assembled as described in section 2.5.6 using the NEBuilder® HiFi DNA Assembly Cloning Kit (E5520). The ligation reaction control was provided by the kit. The no template controls were treated in the same way before being plated onto LB agar or LB agar containing 80 µg/mL chloramphenicol.

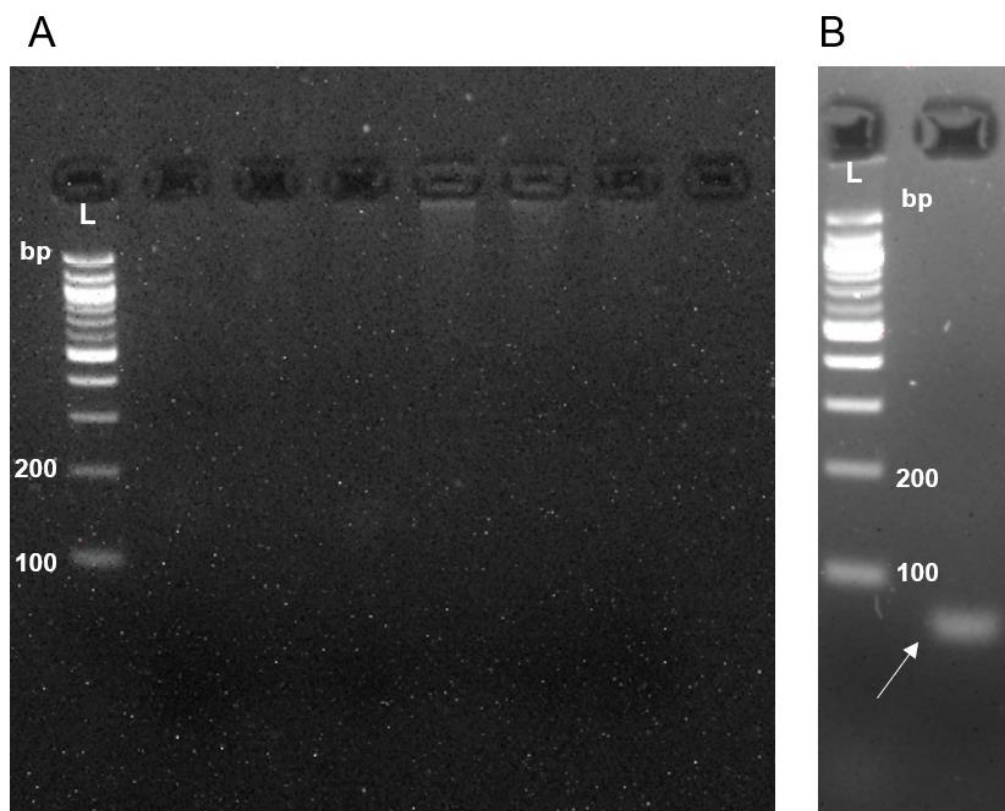


Figure 3.13. The SpyTag wasn't successfully amplified following HiFi DNA assembly. Colony PCR was performed on transformants obtained in 3.5 as described in section 2.5.6. (A) Electrophoresis using 4% agarose gels was performed on all EspC-mCherry-ST and EspC-ST transformants obtained, but no SpyFragment was detected. (B) The SpyTag fragment (~40 bp) has been successfully amplified previously using the same primer pair from pLA33C1ST1 (see Fig. 3.5.). Representative gels are shown (n=3).

3.2.5. The introduction of the TC tag into mCherry

The FIAsh-EDT₂ substrate has been proven to bind the TC motif (CCPGCC), making FIAsh technology likely to have almost no hindrance to protein functionality. It is well known that FP structure is essential to its fluorescence ability. Still, it was decided to investigate whether inserting TC into mCherry (pBAD33mc) might be possible without losing protein function. Even a misfolded mCherry-TC fusion protein which lost its fluorescence should still be detectable with FIAsh-EDT₂ and therefore alleviate the concern EspC patchy localisation is in any way affected by the labelling method. Only one site on mCherry has P and G residues together (at positions 190 and 191 of native mCherry), so the four cysteines were inserted around it. Although the mutagenesis worked, and

the insert was verified by sequencing, the resulting molecule exhibited neither the natural red fluorescence nor the green fluorescence resulting from FIAsh staining (Fig. 3.14., microscopy data not shown).

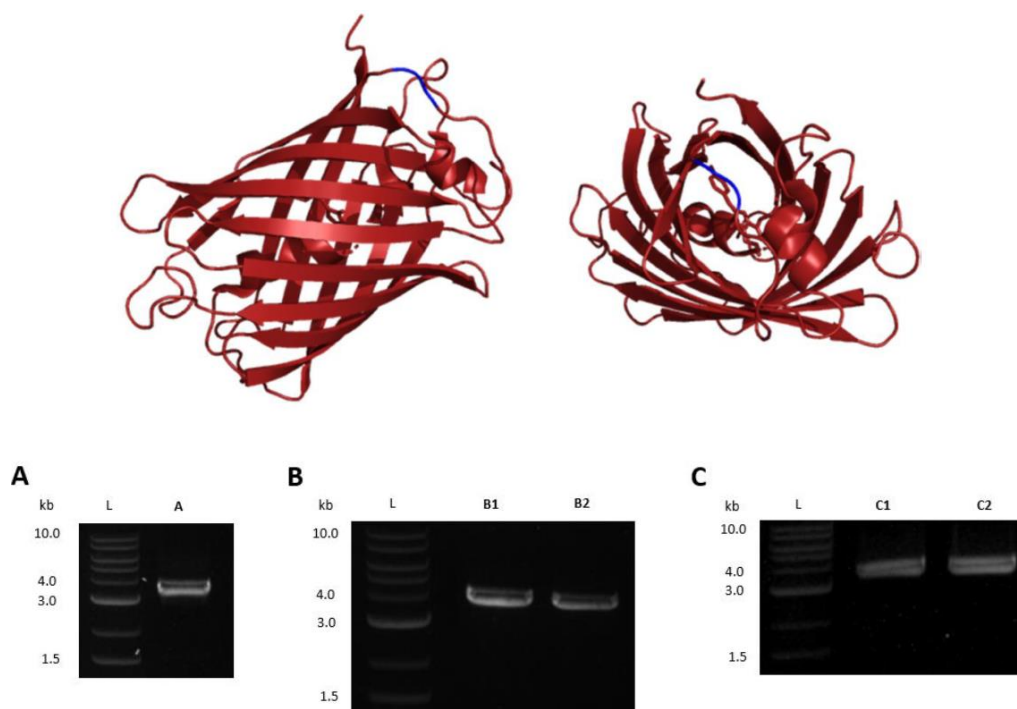


Figure 3.14. A TC tag was successfully engineered into mCherry using site-directed mutagenesis. (Top) The predicted crystal structure of mCherry with the TC tag inserted into an external area (indicated blue on the crystal structure, UniProt ID: X5DSL3). (Bottom) A - Successful SDM using primer pair MS1/2 to insert all four tetracysteines between positions 189 and 192 of mCherry. Transformation using this product was unsuccessful. B – Two successful SDM PCR of B1 (pmCherry DNA, 0.5 μ M primer concentration) and B2 (pmCherry DNA, 1 μ M primer concentration) were performed using primer pair MS3/4 to insert the first two cysteines between positions 189 and 190. B2 was successfully transformed into NEB 5- α Ultracompetent *E. coli* using chemical transformation (described in 2.7.3) to create pmCherry-CCPG. C – Two colonies (C1 and C2) were picked, their DNA extracted and SDM PCR was performed using primer pair MS5/6 to insert the remaining two cysteines between positions 191 and 192 of mCherry. C2 was successfully transformed into NEB 5- α Ultracompetent *E. coli* to create pLA33c1mc-TC. L – ladder. kb – kilo base pairs.

A new site was chosen between S226 and T227 at the C-terminus of the protein, making the tag less likely to interfere with folding and the fluorescence mechanism of mCherry. The mutagenesis did not work, and the site was abandoned. Further inspection revealed that the template plasmid subjected to mutagenesis had been the 3.7 kb pGEMT-mCherry rather than the 6 kb pLA33c1mc. A final attempt was therefore made to introduce the tag after the last residue (K236), ensuring it would not affect protein folding. Due to time constraints following initial unsuccessful SDMs, the experiment was not pursued further.

3.3. Discussion

FPS commonly used in protein localisation have their drawbacks, being bulky and prone to homodimerisation. Even true monomers (like mCherry, derived from dsRed) have now been reported as potentially disruptive to normal protein function and causing clustering artefacts (228). It was, therefore, necessary to validate the microscopy data concerning the localisation of EspC and AaaA ATs, where for the first time, ATs are reported to localise into helical patches along the length of *E. coli* MG1655 cells (206). STSC technology was chosen (see section 1.8.3.) to label native and mCherry-tagged EspC (in higher and lower copy number vectors) and compare their secretion efficiencies by quantifying the fluorescence captured at the OM. The ST consists of 39 nucleotides, making it easy to clone and unlikely to affect AT folding.

The challenge in tagging ATs, especially OM-cleaved SPATEs, is that a tag cannot be placed at either of the termini because the SP is cleaved at the IM, and the translocator is cleaved off at the OM. This is also why the less invasive labelling techniques used for surface-tethered proteins cannot be utilised for ATs such as EspC. SpyCatcher-sfGFP and SpyCatcher-EQ-sfGFP were efficiently purified using Ni-IMAC (Fig. 3.2. and 3.3.). The ST was placed between S349 and I350 of EspC (see Fig. 3.2.), close to where mCherry was previously inserted. The PCR products obtained using the primer pair MS12-13 (see Table 2.3.) could not be sequenced. The insertion site was then moved to the very N-terminus of the passenger that is exposed after SP cleavage. One successful native EspC-ST (pBAD33C1ST1) mutant was obtained, but none for EspC-mCherry were generated. It should be noted that pBAD33C1ST1 was functional in binding SC-sfGFP and successfully fluoresced following the published protocol, opening the way for this construct to visualise secreted EspC in future experiments (Table 3.1.).

The difficulty experienced trying to insert ST into EspC-mCherry using SDM indicated no more genetic manipulation is possible at the site mCherry was successfully inserted (see Table 2.2., Fig. 3.6.). Next, it was

difficult to predict the site where ST would not interfere with SP cleavage (see Fig. 3.7. and 3.9.). The SDM primers generated by NEBaseChanger® yielded mutants with random parts of the *espC* gene inserted instead of the ST, probably owing to the non-specificity of the primers (Fig. 3.10.). Although it was not possible to obtain EspC-mCherry-ST or EspC-ST mutants using HiFi DNA Assembly, this method is probably more likely to work eventually. There was insufficient time to optimise the method, particularly increasing the ligation step's insert fragment - backbone fragment ratio.

For the duration of this process, the aim was to find alternative, more robust evidence that EspC-mCherry fusion proteins are secreted adequately out of the cell. The experimental setup for SR-SIM already uses a 1:10 dilution of the inducer amount generally used (0.2% arabinose, see section 2.15.). Ashawesh (206, 207) used cell fractionation to determine where EspC-mCherry fusion proteins are located and found them only at the OM, with no aggregates detected in any other compartment (207). Additionally, Ashawesh detected EspC-mCherry in cultured supernatants using α -EspC. Using the Yang method, the isolation of inclusion bodies from EspC-mCherry cultures grown for super-resolution microscopy experiments was performed. To date, no insoluble protein fraction has been detected by SDS-PAGE (246).

By incorporating a TC tag into mCherry, the aim was to prove no change in morphology or FP localisation occurs in FIAsh-EDT₂ labelled *E. coli* MG1655. The insertion of TC around P190 and G191 yielded mCherry-TC, but its red fluorescence was lost, proving FP structural integrity is essential to its function (Table 2.2., microscopy data not shown). Due to time constraints, it was not possible to pursue the construction of mCherry-TC further.

Both EspC tagged with large (mCherry) and small (TC) protein tags localise in the same pattern, increasing the likelihood that this is its native localisation. To increase the weight of microscopy data reported by Ashawesh *et al.* (2018), implementing STSC was tried to measure the

secretion efficiency of the fusion protein. However, no functional EspC-mCherry mutants were obtained to compare them with EspC-ST (206). mCherry is still a standard among red FPs, prompting the study to continue with its use to investigate EspC interactions with the actin homologue, MreB (247).

4. EspC's helical localisation depends on the intact cytoskeleton and the Sec machinery

4.1. Introduction

Alongside taking steps towards developing a tool to fluorescently label extracellular EspC (see Chapter 3), this study aimed to track intracellular EspC to elucidate the molecular mechanism of its secretion. The picture of the T5aSS is much clearer today than it was ten years ago, yet it is still unknown how ATs are targeted towards the cell poles or how extracellular release of the passenger occurs (e.g. in SPATEs, see section 1.5.). Reported differences in chaperone dependency of T5a ATs provide another layer of confusion about what exactly goes on in the periplasm (for chaperone dependency and captured interactions, see section 1.5. and Table 1.2., respectively). It is clear ATs rely on essential cell machinery to facilitate OM secretion, like chaperones and the BAM complex. Still, this reliance might extend to the bacterial cytoskeleton and the Sec translocon.

A range of temperature-sensitive (ts) mutants are available for studying Sec-mediated IM translocation, each with a conditional deletion of a sec gene activated by incubation at its non-permissive temperature (see Table 2.1.). Ashawesh *et al.* (2018) have shown that when observing EspC in a SecA temperature-sensitive mutant, instead of the patchy, helical distribution, EspC aggregates at the poles of the cells. Moreover, SecA is required for the correct localisation of a divisome protein DivIVA (246). On the other hand, polar localisation has been reported for the lcsA AT of *S. flexneri* (T5aSS, activates actin polymerases) (29, 206), but it was found to be undisturbed in the SecA_{ts} mutant (115).

The effect of SecA on EspC secretion is not surprising, considering SecA is the motor protein bound to the SecYEG translocase, providing energy (ATP) needed for IM translocation (see section 1.3.1.1.). Several studies have reported the ordered helical patchy arrangement of SecA & SecY (usually bound together at the IM), correlating their localisation to that of

EspC and the Sec translocon (248, 249). Although both Sec and MreB organise into patchy helical structures, they are not a part of the same structure (210, 249).

The spatiotemporal organisation of bacteria is anything but random and is nowadays investigated using FPs. The tubulin homologue FtsZ is the central part of the divisome, forming GTP-dependent protofilaments through GTP-dependent self-polymerisation (169). The protofilaments assemble at their polar end and disassemble at their non-polar end, leaving FtsZ monomers in the middle in a process known as 'treadmilling' (250, 251). FtsZ can use 'treadmilling' to regulate septal PG assembly and complete cell division (252). MreB is another self-polymerising cytoskeletal protein that maintains the rod cell shape by sensing curvature and guiding the insertion of PG material with the rest of the elongasome (see section 1.7.). MreB polymerisation is ATP-dependent, and the resulting protofilaments associate laterally to form a ribbon consisting of two antiparallel MreB protofilaments (173, 253). These filaments are not polar, so their observed motion is not explainable by 'treadmilling' (173, 254). Multiple localisation studies have been carried out to discern the nature of the filaments to enhance our understanding of bacterial growth and division. Many independent studies reported MreB to form 'discrete patches' along the cell (255, 256, 257), while others found MreB to display both 'patchy' localisation and short diagonal bands matching the description of helical protofilaments (172). Ashawesh *et al.* confirmed these reports by showing the helical, patchy localisation using MreB-TC and SR-SIM (206).

The idea proteins are trafficked to the cell poles by interactions with integral membrane proteins is not new (258). MreB has been reported to affect the localisation of polar proteins while itself mostly absent from the poles (176, 259, 260, 261). Strahl *et al.* (2014) showed MreB filaments create regions of increased fluidity (RIFs), interference with which disrupts membrane protein localisation (177, 178, 261).

Considering this, it was decided to attempt to colocalise EspC with MreB. Colocalisation of proteins within bacteria requires single molecule resolution. This can be achieved using super-resolution Structured Illumination Microscopy (SR-SIM). SR-SIM involves the specimen on the coverslip being scanned with three grating angles (rotations) and three phase shifts (phases) to construct a precise location of fluorescent molecules within the bacterium (see Fig 4.1.) (262). Where maximum resolution is required (e.g. protein localisation in bacteria), additional grating angles (five in total) are used at the cost of scanning speed. In turn, decreasing the scan speed can harm SR-SIM algorithms, especially if cell motility and fluorophore photobleaching are of concern (see Fig. 4.1.).

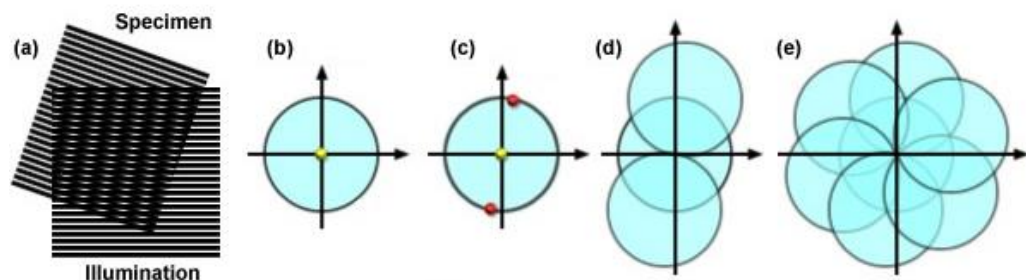


Figure 4.1. Resolution enhancement by Structured Illumination Microscopy (SR-SIM). The diffraction limit is caused by the inability to create objectives large enough to collect high-frequency data (described in 1.8.1.) (a) SR-SIM surpasses the diffraction limit by illuminating the specimen with several patterned gratings and using them to collect high-frequency data from which unresolvable information can be deduced by computer reconstruction. (b) The light blue circle represents the observable space (low-frequency data) in a microscopic sample with the yellow dot denoting the centre of the objective (phase). (c) By illuminating the specimen with a grating, the information is captured from three spots, the central one, and two at the very edge of the observable space (red dots). (d) By superimposing the frequency data obtained from all three spots, algorithms can deconvolve and resolve high-frequency data collected. (e) By increasing the amount angles (rotations) of the grating illumination, more frequency data can be collected to resolve bacterial proteins. This figure has been adapted from SR-SIM Carl Zeiss Microscopy Online lecture (see text).

Even the most advanced microscopy can't capture transient protein-protein interactions (PPIs). In such cases, advances can be made by stabilizing protein partner interactions via crosslinking. There is a range of crosslinking approaches, including exposure to chemicals which bind certain chemical groups of proteins of interest (POIs) (e.g. formaldehyde, glutaraldehyde) (263). Chemical crosslinkers have the limitation of being non-specific since they simply form bridges between specific residues at

a single point in time and, thus, probably don't reflect real interactions (264). The accessory factors to EspC secretion have been investigated by the Hardie group in the past using random transposon libraries (218) and two-yeast hybrid systems (221). While they helped identify potential factors affecting EspC secretion, follow-up studies did not verify these interactions. Therefore, a site-specific approach must be used to limit crosslinks formed with the POI and its interaction partners. Photoreactive unnatural amino acids (UAAs) like p-benzoyl-phenylalanine (pBpa) can be incorporated into the POI by amber suppression. Using recombinant production of plasmids encoding the tRNA_{CUA} reverse synthase and its cognate tRNA_{CUA} in the presence of pBpa, a genetically engineered TAG (amber) stop codon could incorporate pBpa into the EspC sequence during its biogenesis (see section 1.8.4.) pBpa has been tried and tested for studying protein-protein interactions (PPIs) in the EspC EHEC homologue, EspP, which renewed interest in T5SSs after reporting AT chaperone dependency (43).

The findings reported by Ashawesh et al. (2018) strengthened the association between subcellular protein targeting and essential cellular machineries like the Sec translocon and the elongasome (MreB) (206). They proposed a scaffold built by Sec and MreB that 'classical' ATs could use (like tracks) to be delivered to the cell poles and simultaneously become secretion competent. With hopes of providing insight into different stages of EspC secretion, a broader range of sec ts mutants will be used to study the role of SecY (IM channel protein), SecD & SecE (periplasm) in 'classical' AT biogenesis. Colocalisation experiments will be performed between EspC and MreB using SR-SIM fusion proteins constructed by Ashawesh (207). It was hoped to observe them closely associated with the cell membranes and investigate what happens when MreB polymerisation is inhibited by A22. Additionally, 3D SR-SIM will be utilised to construct a complete picture of the shapes and sizes of helical localisation patches. Finally, with amber suppression and incorporation of pBpa to the native EspC sequence, an attempt to identify EspC crosslinked interaction partners *in vivo* will be performed.

4.2. Results

Ashawesh (206, 207) imaged fluorescent EspC and MreB in separate cells and observed matching helical structures but did not co-localise the two proteins in the same bacterium to verify their interaction. The red mCherry-tagged EspC (pLA18C1mc) selected from Ashawesh's collection was used since it exhibits high and consistent expression alongside the green MreB-TC (pLA33mreB-TC). These two were explicitly chosen since mCherry fusion to MreB disrupts its function and changes the cell shape. In contrast, EspC secretion still occurs following insertion of the 27 kDa mCherry as EspC-mCherry fusions are isolated from culture supernatants (207).

To determine whether EspC and MreB interact in the same bacterium, plasmids encoding MreB-TC and EspC-mCherry were introduced simultaneously into *E. coli* MG1655. To accommodate plasmid antibiotic resistance compatibilities, the lower copy number plasmid pBAD33 (Cm^R) was used to produce MreB-TC. In contrast, the higher copy number pBAD18 (Amp^R) was chosen to produce EspC-mCherry. No difference in EspC localisation was observed in previous studies between the production of EspC-mCherry in the pBAD18 or pBAD33 vectors, which have different copy numbers (206, 207).

4.2.1. A range of Sec temperature-sensitive mutants disturb EspC localisation

Patchy helical localisation of EspC is altered when examining EspC-mCherry in the SecA_{ts} mutant (mutation activated at 42 °C). EspC can still cross the IM but localises to the cell poles (206). To determine whether the defect in EspC-mCherry localisation was a common feature of Sec inactivation, it was examined for SecY_{ts}, SecD_{ts} and SecF_{ts} mutants. These mutants are distinguished by their ability to limit protein secretion when incubated at restrictive temperatures. Complete deletion of Sec components would result in inviable cells since essential genes require Sec-mediated translocation (265). It should be noted that due to the mutations being ts genetic lesions causing the loss of protein function, the phenotype can also 'leak' at the permissive temperature.

The EspC-mCherry encoding plasmid pLA33C1mc was introduced into each mutant. Bacteria were cultured as described in section 2.4.1. and imaged on the Zeiss Elyra PS.1 (see section 2.16.). *E. coli* MG1655, also containing EspC-mCherry (pLA33C1mc), was subjected to the same protocol as the ts mutants to compare cell viability, fluorescence signal strength and the effect of high and low temperatures on EspC localisation.

E. coli MG1655 cells producing EspC-mCherry (pLA33C1mc) showed the patchy helical localisation of EspC, which did not alter in the higher or lower temperatures used to activate the Sec mutations: 42 °C for SecA_{ts} & SecY_{ts} and 25 °C for SecD_{ts} & SecF_{ts} mutants (Fig. 4.2.). When the mutations were induced for SecA (a motor protein on the IM side) and SecY (IM channel), EspC-mCherry localised to the cell poles (Fig 4.2.). The bright red dots outside SecA_{ts} cells are fluorescent microspheres that calibrate imaging settings and have no biological relevance. The mutations in *secD* & *secF* (encoding proteins located on the periplasmic side of the IM) inhibited the detection of EspC secretion entirely (Fig. 4.2.). All imaging of the Sec TS mutants was performed for a minimum of three technical and biological replicates.

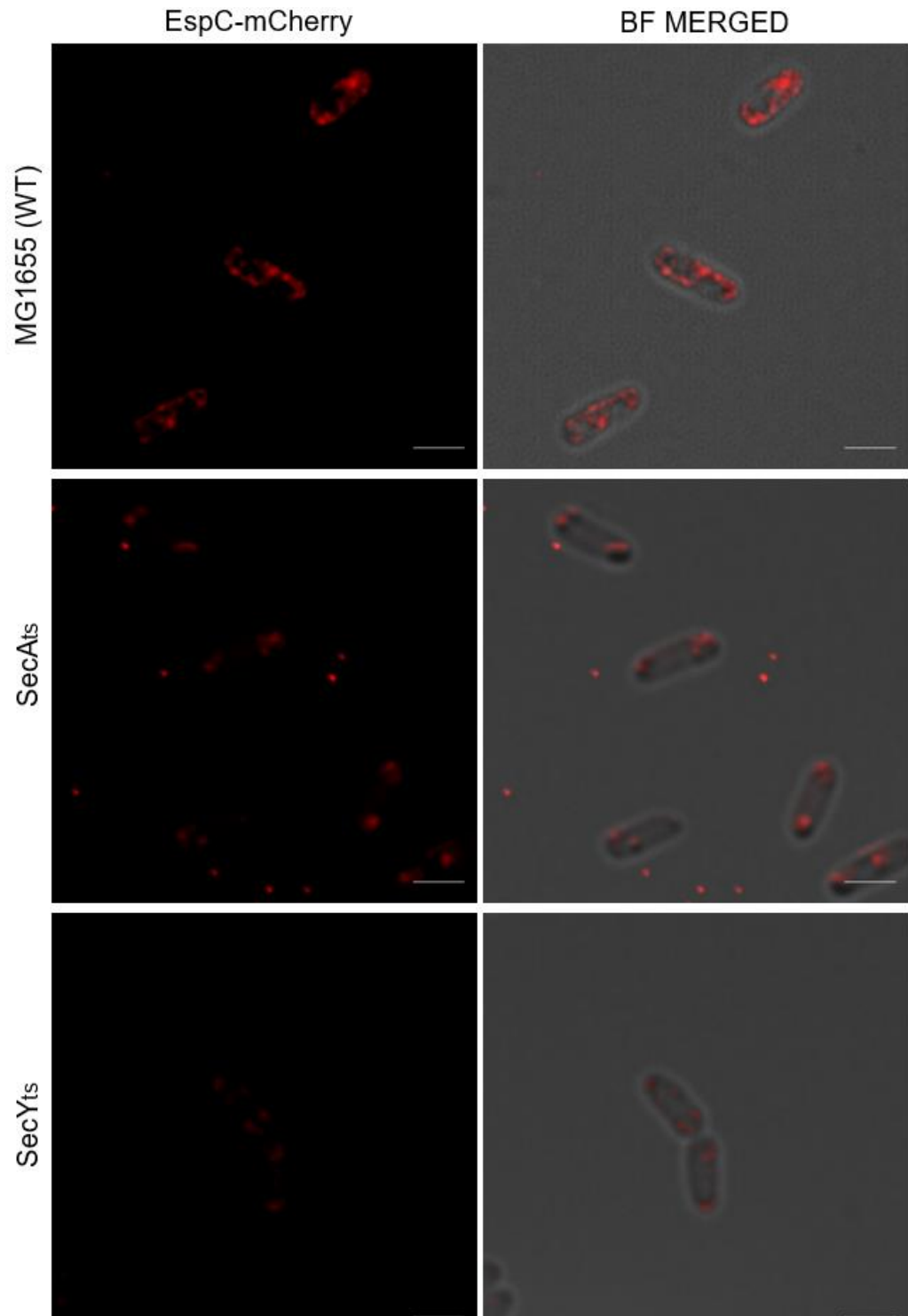


Figure 4.2. EspC-mCherry localisation is not disrupted at high temperatures (42° C) in *E. coli* MG1655, but its localisation is altered in SecA and SecY mutants at 42° C. The wildtype MG1655 and TS mutants of SecA & SecY containing the EspC-mCherry encoding plasmid are shown. Overnight cultures were diluted to OD₆₀₀ 0.05, induced with 0.02% arabinose until OD₆₀₀ 0.3 and incubated at 30° C. A temperature switch was performed to activate the TS mutations. All strains (including the WT) were incubated for an additional 3 h at the non-permissive temperature (25° C). The bright red spots outside SecA_{ts} are fluorescent microspheres used to calibrate SR-SIM settings. The scale bar represents 2 µm. TS – temperature-sensitive; BF – bright field.

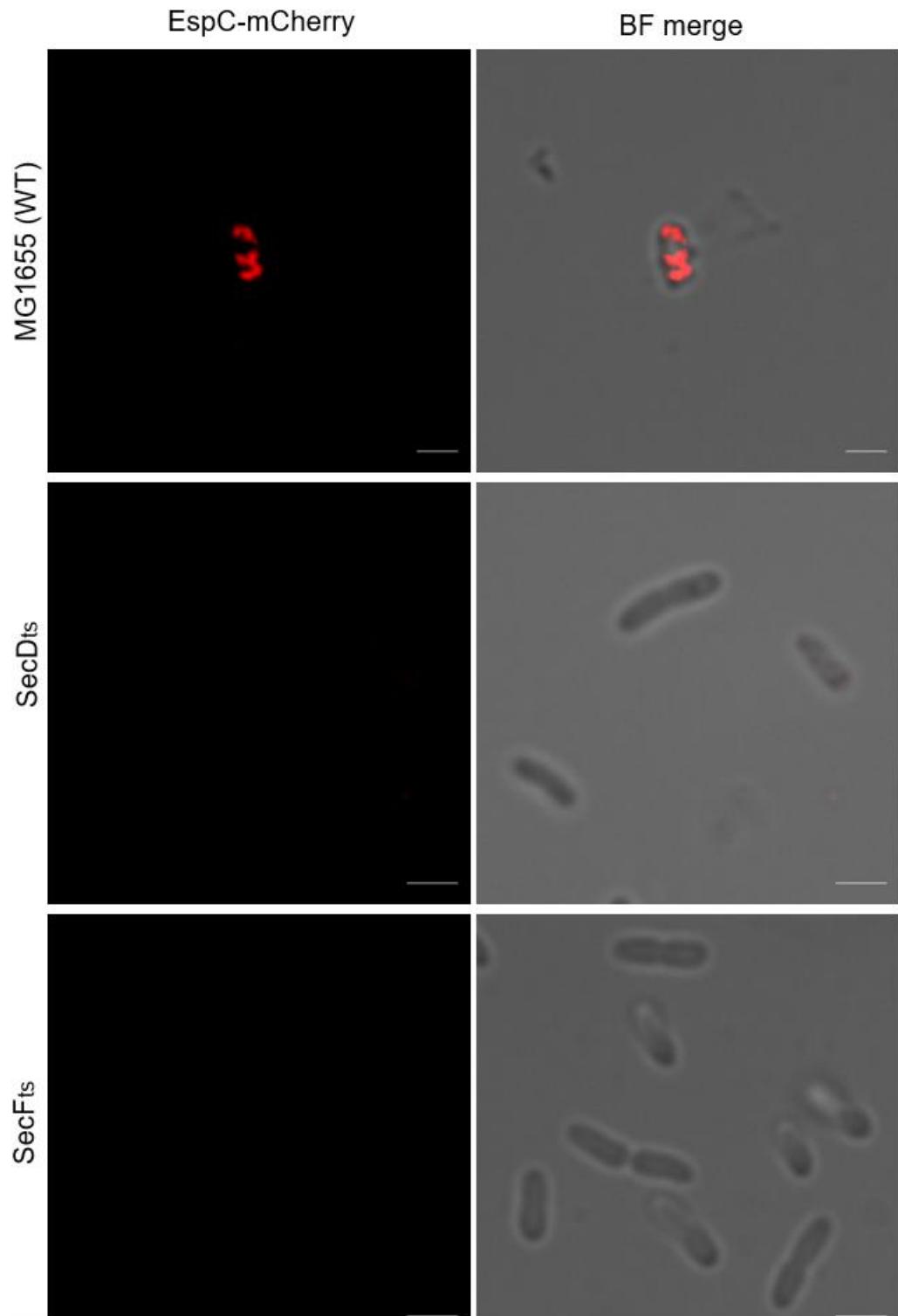


Figure 4.3. EspC-mCherry localisation is not disrupted at low temperatures (25° C) in *E. coli* MG1655, but it is not detectable in SecD and SecF mutants at 25° C. The wildtype MG1655 and TS mutants of SecD & SecF containing the EspC-mCherry plasmid were analysed. Overnight cultures were diluted to OD₆₀₀ 0.05, induced with 0.02% arabinose until OD₆₀₀ 0.3 and incubated at 37° C. A temperature switch was performed to activate the TS mutations. All strains (including the WT) were incubated for an additional 3 h at the non-permissive temperature (25° C). The scale bar represents 2 µm. TS – temperature-sensitive; BF – bright field.

4.2.2. SR-SIM confirmed that EspC and MreB colocalise

The resultant *E. coli* MG1655 containing EspC-mCherry (pLA18C1mc) and MreB-TC (pLA33mreB-TC) was grown overnight in LB containing appropriate antibiotics. Protein production was induced by adding 0.02% L-arabinose, and the bacterial cultures were incubated for 3 h (~0.3 OD₆₀₀). The MreB-TC tag was activated by adding FIAsh-EDT₂, as described in 2.13. and the cells were imaged on 1.2% agarose pads using MatTek glass dishes (see sections 2.16. and 2.16.1.) using SR-SIM. Figure 4.4. shows a population of the bacteria, and it is clear that MreB-TC had a weaker fluorescent signal than EspC-mCherry.

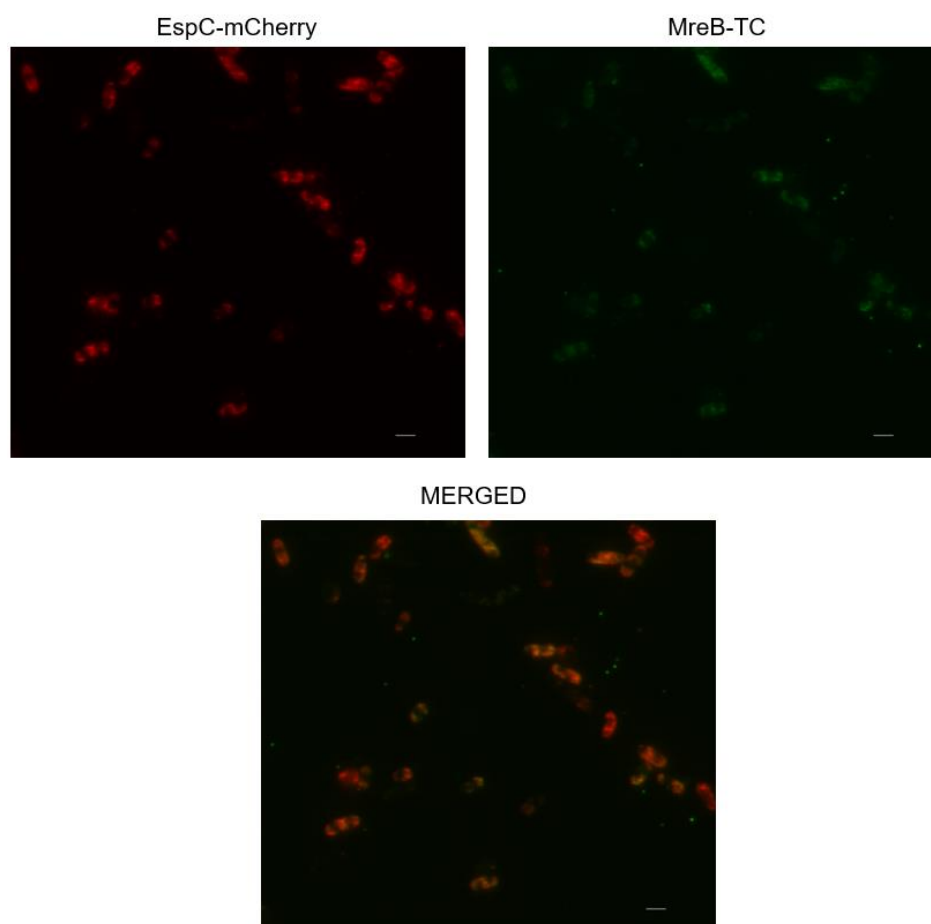


Figure 4.4. Initial indication that EspC interacts with MreB. An unprocessed image of EspC-mCherry (red) and MreB-TC (green) in live *E. coli* MG1655(pLA18c1mc, pLA33mreB-TC) cells to provide a population view. The cells were prepared as described in 2.16. and imaged on the Zeiss Elyra PS.1 C-Apochromat 63x/1.2W Korr M27 water immersion. Colocalisation is indicated by reddish orange, since photobleaching of the green MreB-TC reduced the level of yellow tones. The top two panels show the red channel on the left (EspC-mCherry) and green channel on the right (MreB-TC) with the merged image below. The bright green dots are 1 μ m fluorescent beads used to align the red and green fluorescent channels. The scale bar represents 2 μ m.

On closer inspection of individual cells at higher magnification (Fig 4.5.), it was evident that not all the bacteria produced a similar level of green fluorescence, suggesting MreB-TC had not uniformly interacted with the FIAsh-EDT₂ substrate. The advantage of the differential MreB-TC levels was the verification that the green signal is not a product of fluorescence bleedthrough between the channels since bacteria lacking green fluorescence could be identified (Fig. 4.5.).

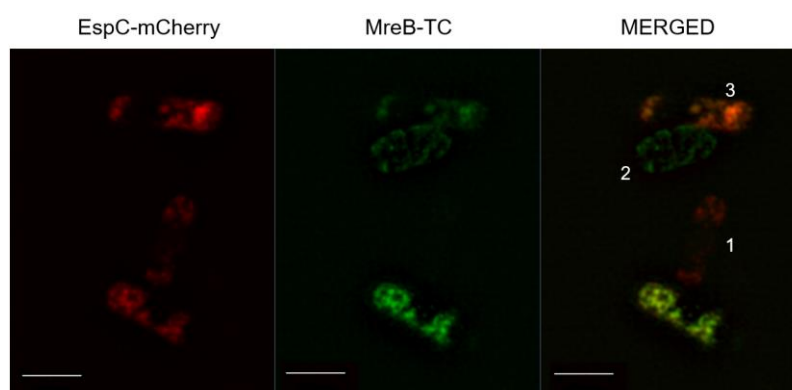


Figure 4.5. Live *E. coli* MG1655 simultaneously harbouring plasmids encoding EspC-mCherry and MreB-TC do not consistently exhibit the expected red and green fluorescence, suggesting the two fluorophores are detected as separate signals. A selection of individual bacteria from Fig 4.1 were processed as described in 2.14. to increase their resolution. The three distinct phenotypes evident are indicated as: 3 - bacterium exhibiting both fluorescence signals (orange/yellow). The cells were prepared as previously described (see sections 2.16. and 2.16.1.), 2 - bacterium with only MreB-TC detectable (pLA33MreB-TC, green) and 1 - bacterium with detectable EspC-mCherry only (pLA18c1mc, red). The scale bar represents 2 μ m.

Improving the resolution of the imaging by applying 2 Dimensional SIM revealed that in all *E. coli* cells exhibiting detectable fluorescence originating from both plasmids, it was found that EspC and MreB strongly colocalise with PCC = 0.968 (Pearson's correlation coefficient, ranges from -1 for no correlation to 1 for perfect correlation) (Fig. 4.6.A.). This colocalisation isn't entirely homogenous, as there are areas of discrepancy where one protein is detectable and the other is not (Fig. 4.6.B., 4.7.). However, overall, the two proteins appear to be in the same places throughout the cell and distributed in a helical pattern (see section 4.3.).

A 3D analysis was performed to establish if the localisation patterns of EspC-mCherry and MreB-TC follow a helical path. Unexpectedly cell-flattening was observed, making rendered 3D analyses technically impossible (Figure 4.7. A-D). This flattening was observed in all samples (n=3) and independent repeats of the experiments (not shown). To determine whether the cell shape derived from the combination of the two proteins or could be attributed to only one, they were produced and imaged similarly. All bacteria containing the mCherry-EspC fusion exhibited flatness, while EspC-TC and MreB-TC separately did not cause the same effect (206).

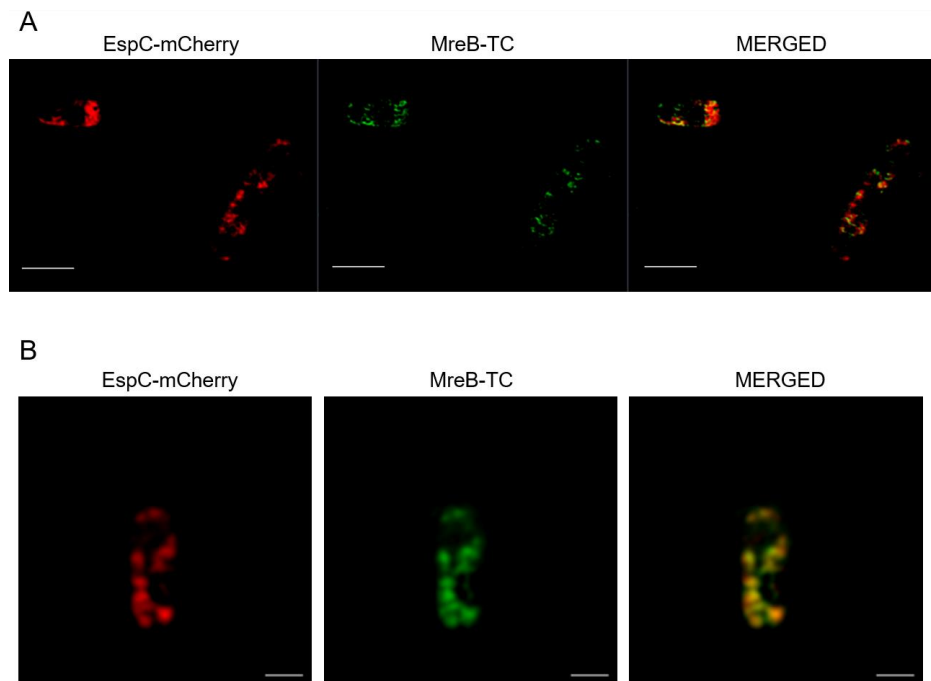


Figure 4.6. 2-dimensional SIM confirmed that EspC-mCherry and MreB-TC colocalise in discrete patches around the *E. coli* MG1655 cell. The cells were grown and imaged as described before (see section 2.16. and 2.16.1.). (A) 2D Structured Illumination Microscopy was performed and analysed using Zeiss Elyra PS.1 and ZEN Black (sectioning: 99; raw scale; noise filter - 5). (B) EspC-MreB colocalisation on the single cell level. The scale bar represents 1 μ m.

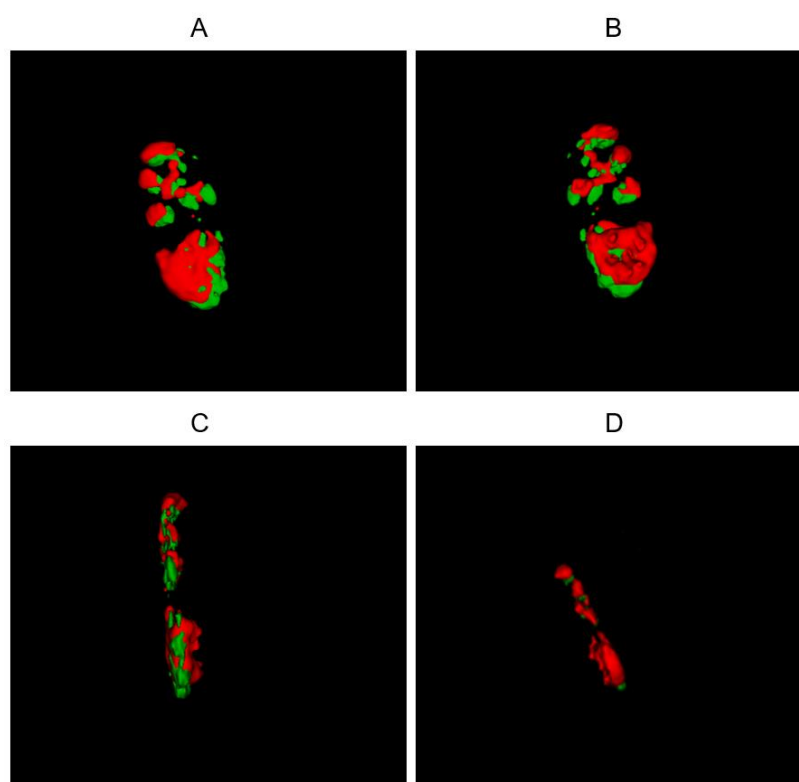


Figure 4.7. 3-dimensional super-resolution Structured Illumination Microscopy reveals *E. coli* MG1655 cells producing EspC-mCherry (red) and MreB-TC (green) exhibit cell flattening. For this data set, fluorescent beads were used to verify the cell shape and elongation, and to correct the channel drift/shift (TetraSpeck™ microspheres 1 μm). A – a single cell processed using 3D SR-SIM shows remarkably similar and closely associated localisation (facing the coverslip, en face). In places where the two proteins overlap, the stronger signal is shown by the 3D rendition depending on the angle of observation B – the point of view is flipped 180° to show the back of the cell. C and D show the localisation on the left and right profiles, respectively. The flattening effect (collapse) has been consistent throughout colocalisation experiments. The slight displacement of the two fluorophores in relation to each other is thought to be more representative of real-life PPIs (see section 4.3.).

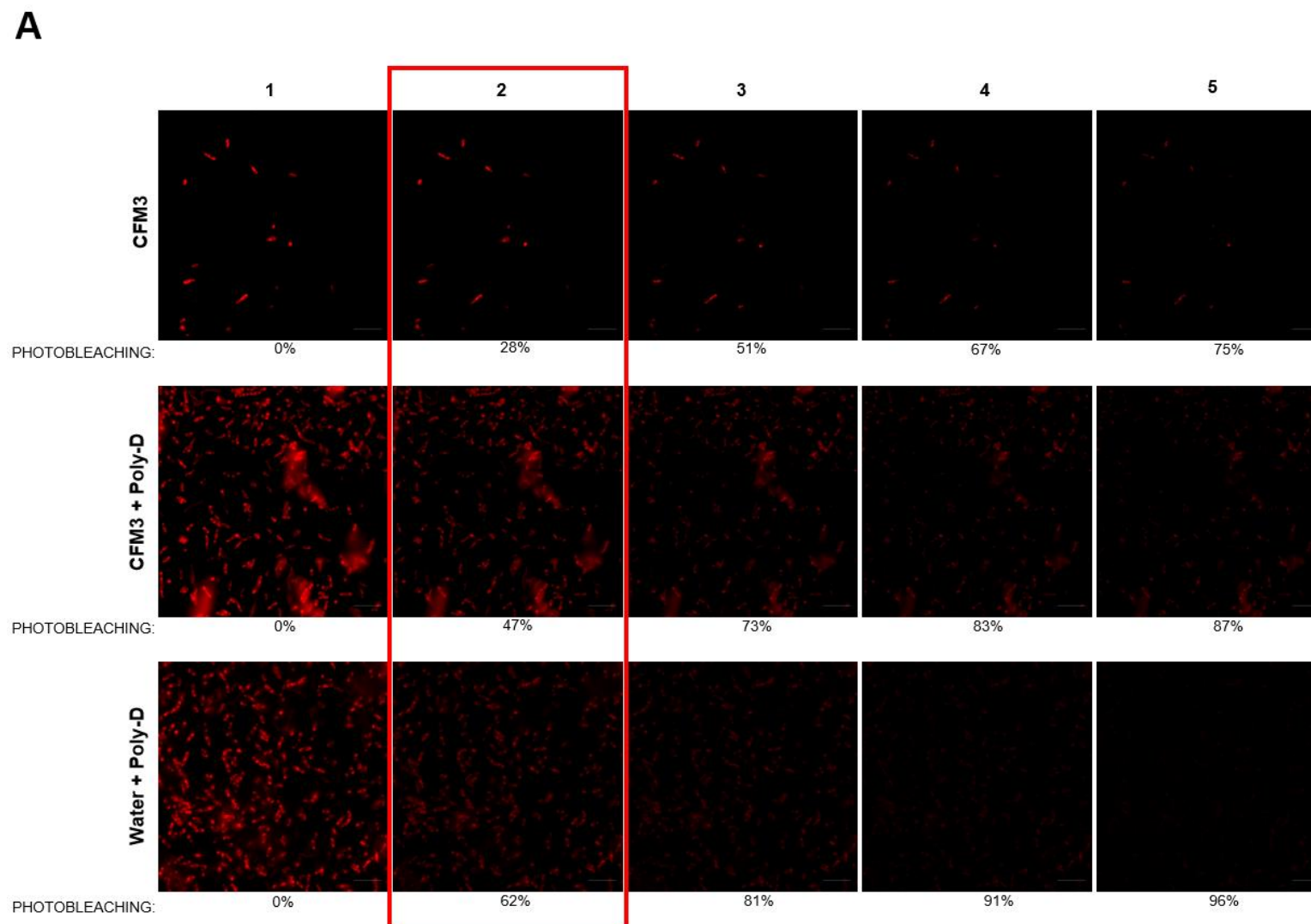
4.2.3. Optimisation of SR-SIM image acquisition for 3D imaging

To overcome the diffraction limit of light microscopy (discussed in 1.8.2.), SR-SIM was used to illuminate the localisation of EspC with MreB in *E. coli* MG1655 (see section 4.2.). The low fluorescence signal emitting from MreB-TC in relation to EspC-mCherry and the 'collapsing' morphology observed in every 3D-processed cell were reported above (see section 4.2.). Furthermore, cell movement was observed, which negatively impacts the precision of the slow-paced SR-SIM scanning. Cell motility makes it impossible to acquire a detailed cell structure, especially when cell slices are needed for 3D analysis – it results in blurriness, as was observed in 4.6.B. Therefore, it was decided to swap the agarose pad mounting method and optimise signal acquisition by using high refractive index anti-bleaching medium CFM3 (CitiFluor™). CFM3 was also tried in combination with Poly-D-Lysine, which is used to facilitate cell adhesion to coverslips. Lastly, cells mounted in Poly-D-Lysine mixed with water were imaged for comparison. Live *E. coli* MG1655 cells were cultured & mounted as described in 2.16. and 2.16.1., and examined on the Zeiss Elyra PS.1. EspC-mCherry Cells treated with CFM3 were not prone to bleaching, showing a gradual drop reaching 75% photobleaching after the fifth rotation (Fig. 4.8.A.). Still, nearly all cells in a region of interest were motile and scarce, making it unsuitable for imaging cells in 3D (Fig. 4.8.B.). In contrast, EspC with Poly-D-Lysine/CFM3 loses nearly 50% of its signal intensity after the first rotation at the expense of 3D imaging quality (Fig. 4.8.A.). Similarly, without the presence of the anti-bleaching agent, cells mounted in Poly-D-Lysine/water bleached 67% after the first rotation, making the images unusable for super-resolution processing. On the other side, only a small amount of cells treated with either Poly-D-Lysine/water or Poly-D-Lysine/CFM3 showed any cell motility, proving Poly-D-Lysine is an effective surface adhesive (Fig. 4.8.B.). Finally, it was decided to use 0.1% glutaraldehyde to see if it could work as a suitable substitution.

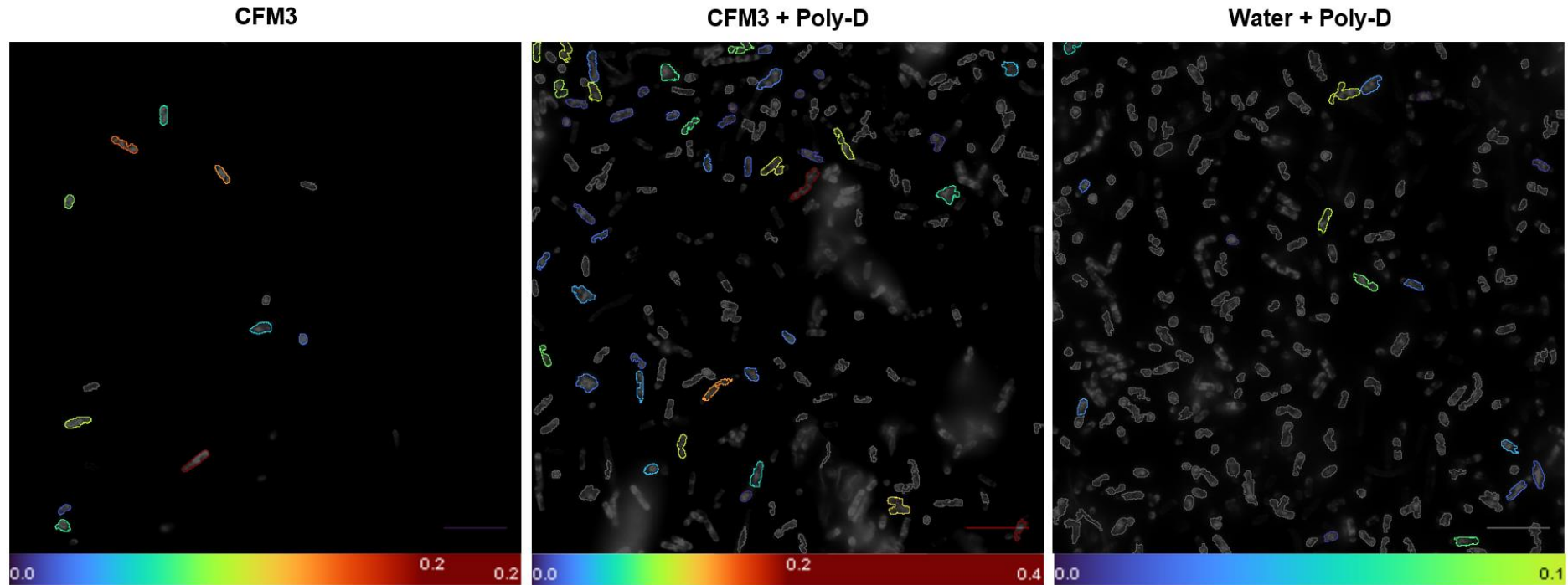
Figure 4.8. Brownian motion can be minimized by applying the adhesive polylysine to improve live 3D SIM imaging of EspC-mCherry, but it reduces the effectiveness of the antibleaching agent CFM3.

Three mounting methods were used to compare their adherence and imaging qualities for use in live 3D scanning of EspC-mCherry localisation (pLA33C1mc). High-refractive and anti-bleaching CitiFluor™ CFM3 mounting medium was used alone and in combination with Poly-D-Lysine (Sigma).

(A) The extent of photo-bleaching was calculated using the difference in fluorescence intensity measured after each SIM rotation (1 – 5, see text) when compared to the fluorescence intensity recorded after the first rotation. The scale bars represent 10 µm.



Cont. on next page→



cont. **Fig. 4.8. (B)** The use of Poly-D-Lysine as a coverslip adhesive successfully prevents Brownian motion in the samples when compared to the cells mounted with the photo-stable CFM3. The TrackMate ImageJ plugin was used to calculate the movement of bacteria between SIM rotations when mounted with/without CFM3/Poly-D-Lysine. The grey-outlined cells are static. Cells outlined in colour moved during image acquisition, and the distance travelled is rated on a scale from 0.0 to 0.4 μm (max. recorded drift) using a colour spectrum. Blue indicates minimal/no movement and dark red indicates movement of 0.2 μm or higher. The scale represents 10 μm . The results were verified in a minimum of three technical and biological replicates.

4.2.4. EspC-mCherry remains partially colocalised with MreB-TC when MreB polymerisation is inhibited by A22

To investigate whether MreB polymerisation was required for interaction with EspC, the chemical inhibitor of MreB polymerisation, A22, was added to growing cells of *E. coli* MG1655 producing EspC-mCherry (from plasmid pLA33C1mc) and MreB-TC (from plasmid pLA33mreB-TC). The samples in the images shown in Fig. 4.9. were acquired before the systematic optimisation of preparing SR-SIM samples described in 4.2.3. The second one was undertaken after the switch to 0.1% glutaraldehyde (see below, Fig. 4.10-11). The cells were cultured as described in 2.14. Images were taken by the Zeiss Elyra PS.01. As expected, the addition of A22 results in the circularisation of the cell since there are no actin filaments formed by MreB to maintain the rod shape. It was hypothesized that since the MreB structure was disrupted, EspC-mCherry would diffuse freely in the periplasm. However, surprisingly, EspC-mCherry remained associated with MreB, with the two proteins accumulating adjacent to each other (see orange-yellow colour indicated by white arrows in the merged panel of Fig. 4.9.).

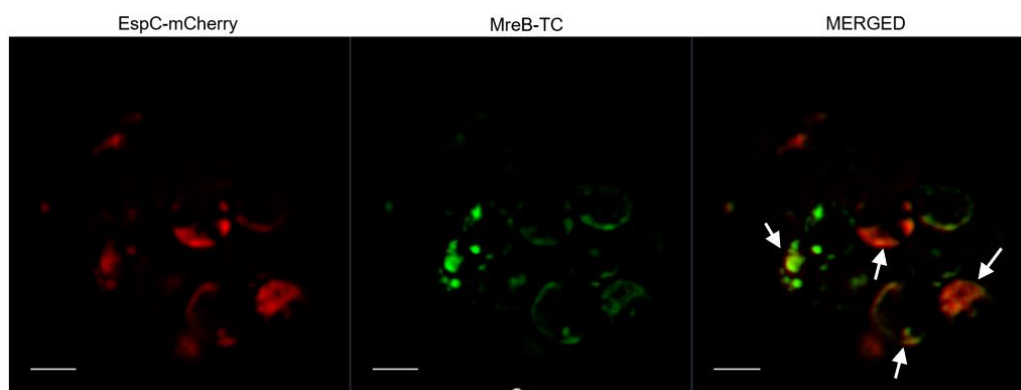


Figure 4.9. EspC-mCherry colocalises with MreB-TC after inhibition of MreB actin polymerisation with A22 (2D SR-SIM). *E. coli* MG1655 containing both EspC-mCherry (pLA18C1mc) and MreB-TC (pLA33mreB-TC) was grown overnight in LB containing appropriate antibiotics. One mL of culture was added to 99 mL LB containing antibiotics and 0.02% arabinose. The culture was shaken at 37 °C (200rpm) for 1 hour, followed by the addition of 10 µg/mL A22. Subsequently, the cells were incubated for an additional 3 h, harvested by centrifugation, washed with ice cold PBS and resuspended to ~0.5 OD₆₀₀. The cells were observed under the Elyra super-resolution microscope and processed using SR-SIM as before (see section 2.16.). On the left (panel A), the red fluorescence representing EspC-mCherry is shown with the green fluorescence of MreB-TC in panel B and the merged image in Panel C. The scale represents 2 µm. Representative sample of three repeats is shown. Asterisk indicates yellow fluorescence of co-localisation.

The examined cells emitted low fluorescence signals and were clumped together and rounded, consistent with the presence of the A22 inhibitor. Although it wasn't possible to assign any biological significance to the observations at this point or scan the cells in 3D, as had previously been observed, MreB-TC appeared not to be distributed in a helical structure. To improve the chances of capturing interactions between EspC-mCherry and MreB-TC in greater resolution, imaging of bacteria fixed with 0.1% glutaraldehyde (GA) for 10 min was performed. The cells were washed and mounted in the high refractive index anti-bleaching medium CFM3 (CitiFluor™), as described in 2.15.1. to reduce TC photobleaching. Image acquisition took place as before. Structured Illumination Microscopy was used to render a 3D model of the A22-treated cells (Fig. 4.10.). As expected, MreB-TC remained localised at the IM. At the same time, EspC-mCherry localised to the IM (colocalising adjacent to MreB) and diffusely spread within the cell. It would seem likely that the secretion of EspC-mCherry was attenuated (Fig 4.10.).

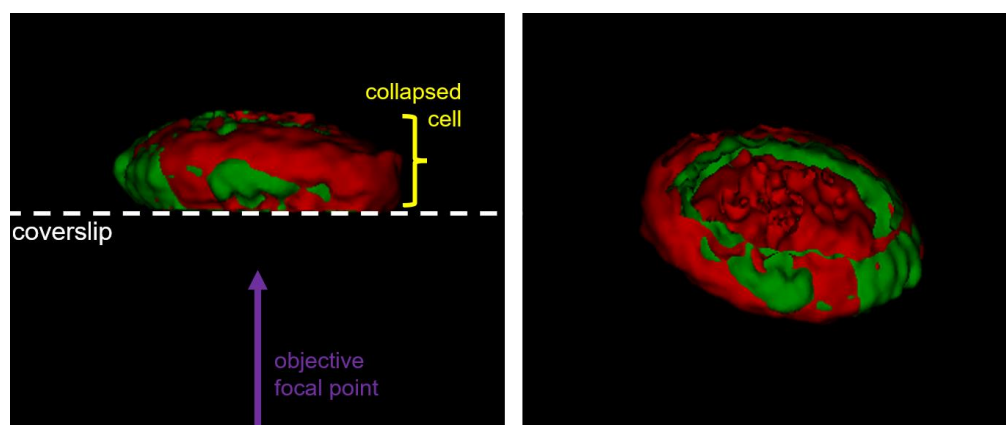


Figure 4.10. 3D SR-SIM analysis of fixed *E. coli* MG1655 reveals EspC-mCherry (red) partially localises adjacent to MreB-TC (green) at the IM after the treatment with A22. A 3D cell is orientated in relation to its position on the coverslip (dotted white line) and where the focal point of the objective was (direction of the arrow) during 3D SR-SIM image acquisition. As demonstrated previously, all imaged cells containing the two plasmids are collapsed/flattened (yellow text). *E. coli* MG1655 containing EspC-mCherry (pLA18C1mc) and MreB-TC (pLA33mreB-TC) was grown overnight in LB containing appropriate antibiotics. The overnights were diluted 1:100 and grown in LB containing antibiotics and 0.02% arabinose. The culture was incubated for 1 h at 37°C with shaking (200 rpm), after which 10 µg/mL S-83,4-dichlorobenzene-isothiourea (A22) was added to prevent MreB polymerisation. The cells were incubated for an additional 3 h, and 1 mL corresponding to 1.0 OD₆₀₀ was harvested. The cells were treated with 0.1% glutaraldehyde for 10 min and mounted with CFM3 as described in section 2.15.1.

As before, there was deformation of the bacteria to create slightly flattened cells which can't be used to investigate the helical patterns of EspC-mCherry and MreB-TC, with cells averaging $<0.5\ \mu\text{M}$ (expected $\sim 0.7\ \mu\text{M}$). Bacteria containing only MreB-TC (pLA33MreB-TC) were cultured in parallel to check for green-to-red fluorescence 'bleedthrough' during the imaging (Fig 4.11.).

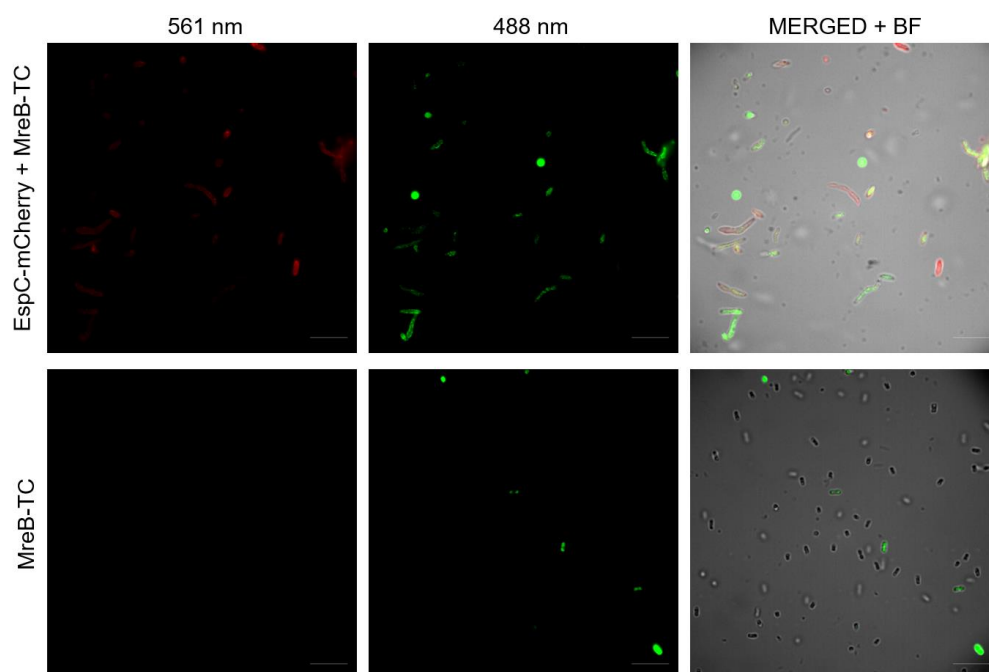


Figure 4.11. There is no fluorescence bleedthrough from the green (488 nm) to the red (561 nm) channel caused by A22-treatment and cell fixation. EspC-mCherry and MreB-TC localisation comes from two separate fluorophore signals, as opposed to MreB-TC causing pseudo-fluorescence in the red (561 nm) channel. EspC-mCherry (pLA18C1mc) and MreB-TC (pLA33mreB-TC) were grown together in *E. coli* MG1655, harvested, fixed and imaged as above (see Fig. 4.10.). MreB-TC was also grown by itself for comparison. The scale represents $5\ \mu\text{m}$. BF – bright field; nm – nano meter.

4.2.5. *In vivo* photo-crosslinking of EspC

In addition to investigating the bacterial molecular machines involved in IM translocation and the maintenance of cell shape hypothesized to play a role in EspC localisation during secretion, a less directed approach was undertaken. EspC interactions have been investigated in the past using a random transposon library (218) and the yeast two-hybrid system (221). Although potential candidates have been identified, the interactions were not backed by follow-up studies. Here, to identify interaction partners of EspC that have hitherto not been discovered and confirm those hypothesised (e.g. MreB, periplasmic chaperones), the first step was to stabilize the interactions using photo-crosslinking with a genetically encoded unnatural amino acid (UAA). This technology uses amber codon suppression to crosslink p-benzoyl-phenyl-alanine (pBpa) when activated by UV light (365 nm). The amber codon TAG is the least used by *E. coli* and does not terminate essential genes, resulting in no effect on cell growth in the absence of pBpa (266). The vector encoding pBpa's tRNA and reverse synthetase pair in a compatible pULTRA vector was kindly gifted to us by Peter Schultz.

Ashawesh (206, 207) had attempted to use the UAA technology for bioorthogonal fluorescence microscopy (using click chemistry and the UAA N ϵ -acetyllysine), so a suitable construct (pLA33C1-TAG₉₅₁) was available with the TAG codon inserted at position 951 of the *espC* ORF.

4.2.5.1. Strains were constructed to enable pBpa photo-crosslinking

The plasmid containing the tRNA synthetase/tRNA orthogonal pair (pULTRA-pBpaRS) for the *in vivo* incorporation of the photo-crosslinkable pBpa (p-benzoyl-L-phenylalanine, Merck) (197) was transformed into *E. coli* DH10B alongside a plasmid encoding EspC_{TAG951} containing the amber stop codon in place of residue 951 (pLA33C1-TAG₉₅₁). Two negative control strains were also prepared in the same background *E. coli*, one lacking the EspC encoding plasmid and the other with a plasmid encoding the native EspC (pLA33C1).

Trial	#1 Native protein	#2 Background UAG	#3 No pBpa control	#4 Photo- crosslinking
expression vector	pLA33C1	pLA33C1- TAG ₉₅₁	pLA33C1- TAG ₉₅₁	pLA33C1- TAG ₉₅₁
pULTRA- pBpaRS	x	x	✓	✓
1 mM pBpa	x	x	x	✓
antibiotics	40 µg/mL Cm	40 µg/mL Cm	40 µg/mL Cm, 50 µg/mL Sm	40 µg/mL Cm, 50 µg/mL Sm

Table 4.1. Table outlining the constructs and growth conditions used to perform pBpa photo-crosslinking. The method was adapted from Farrell and Toroney (222).

4.2.5.2. Optimisation of pBpa photo-crosslinking

An outline of the investigation conducted to assess pBpa incorporation is shown in Fig. 4.11. The contents and treatment of the four parallel cultures assessed are shown in Table 4.1. Of note, the strain containing both the plasmids encoding EspC_{TAG951} plus the plasmid bearing the tRNA synthetase/tRNA orthogonal pair (pULTRA-pBpaRS) was set up in duplicate, and only one version of these included the exogenous addition

of pBpa and was subjected to photo-activation. All cultures were incubated for 20 h before the inducers were added. Following an additional 20-hour incubation, the pellets were harvested in 50 mL aliquots and frozen at -80 °C or resuspended in 12 mL PBS immediately. Only the pellet from flask #4 underwent UV photo-crosslinking for 0, 30, 60 or 120 min (3 mL aliquots). Lastly, the crosslinked samples (along with samples from control flasks) were examined on SDS-PAGE followed by Western blotting with α -EspC (see section 4.7.). The method and materials used for photo-crosslinking are elaborated in more detail in section 2.11. Modifications to the original protocol published by Farrell *et al.* (267) are summarised below. Glycerol minimal media was prepared as described, excluding the addition of heavy metals. Stocks of L-leucine and D-biotin were made in 1 M HCl and water (with a drop of 1 M NaOH), respectively. The final concentration of these in the glycerol minimal media was < 0.02%.

To optimise the crosslinking, several alterations were performed. Since precipitation of pBpa occurred in flask #4, the methodology was developed such that PbpA was added drop-by-drop while swirling, ensuring both pBpa and the flask were prewarmed at 37 °C for 1 hour (268). The growth rate of flasks containing two plasmids (flasks #3 and #4) was improved by normalising overnight cultures to 0.03 (instead of 0.05) OD_{600nm}, and the induction was performed when they reached 0.2 OD_{600nm} (instead of ~0.6), as advised by Ieva (Fig. 4.11.B.) (268). For UV activation of pBpa, lidded Pyrex® glass Petri dishes were used as a UV filter to block bactericidal rays and minimise cell death (Fig. 4.11.D.) (269). The UV lamp was turned on 15 min before the experiment to stabilise at the right wavelength (268). Ice gel packs were used to keep the samples cool throughout the UV treatment and prevent evaporation.

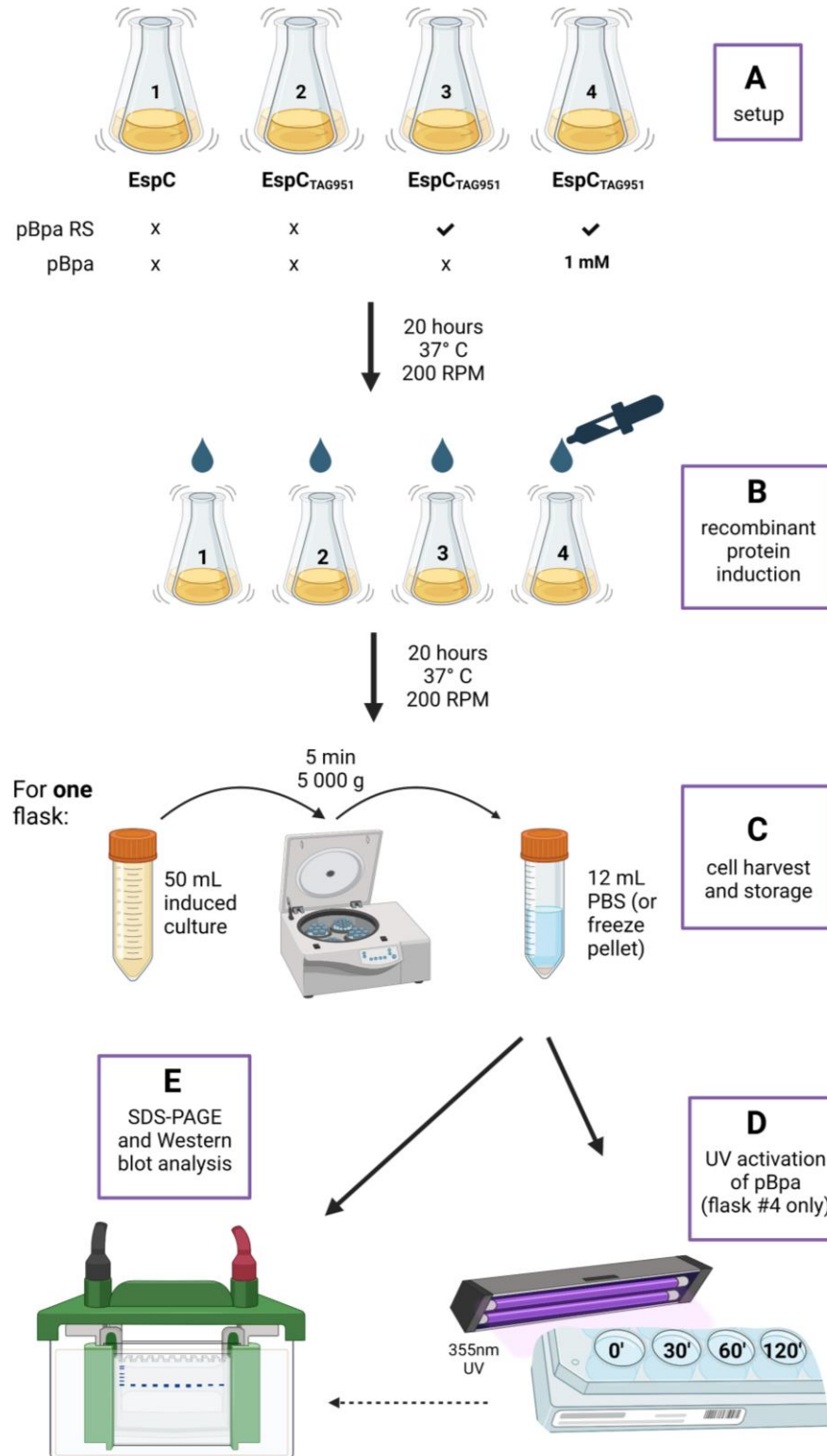


Figure 4.12. Outline of the experimental procedure to photo-crosslink EspC to proteins it interacts with using pBpa. Following recombinant protein production (A & B), 50 mL aliquots were pelleted for long-term storage or immediate use. The pellet from flask #4 was resuspended in 12 mL PBS, divided among four wells and underwent UV treatment to activate pBpa and facilitate crosslinking (up to 2 h irradiation time). The crosslinking products and samples from control flasks were visualised using SDS-PAGE and Western blotting. See section 4.8. for the immunoprecipitation of crosslinked products. The complete method is stated in section 2.11.

4.2.5.3. Further optimisation of protocols to resolve photo-crosslinked products is required

Analysis of crosslinked products using the published protocol by Farrell *et al.* (267) was not ideal. The combination of α -EspC probing of a PVDF membrane during the Western blotting was difficult to analyse due to a high level of background luminescence. The production of full-length (140kDa) and secreted (110kDa) EspC in flask #1, pLA33C1, was confirmed. The lack of evidence of crosslinked products was attributed to the precipitation of pBpa. After the optimisation of sample preparation (see section 4.2.5.1.), the PVDF was exchanged with a nitrocellulose membrane, NuPage 3-8% gels and a high molecular weight ladder (26625, Thermofisher) was used to improve the SDS-PAGE resolution since expected crosslinking products are likely to be bigger than EspC (>110 kDa), and the volume of sample loaded was reduced from 25 μ L per well to 5 μ L per lane (Fig. 4.13.). Although a high molecular weight smear of proteins indicated crosslinking had occurred in the photo-activated samples, no discernable proteins could be further analysed. These samples did contain a unique protein (~80 kDa). However, this size did not correspond with the expected proteins and was too small to be a crosslinked complex (Fig. 4.13.). Moreover, the control flasks (#1-3) did not produce the expected EspC proteins, indicating that further optimisation of this analysis is required.

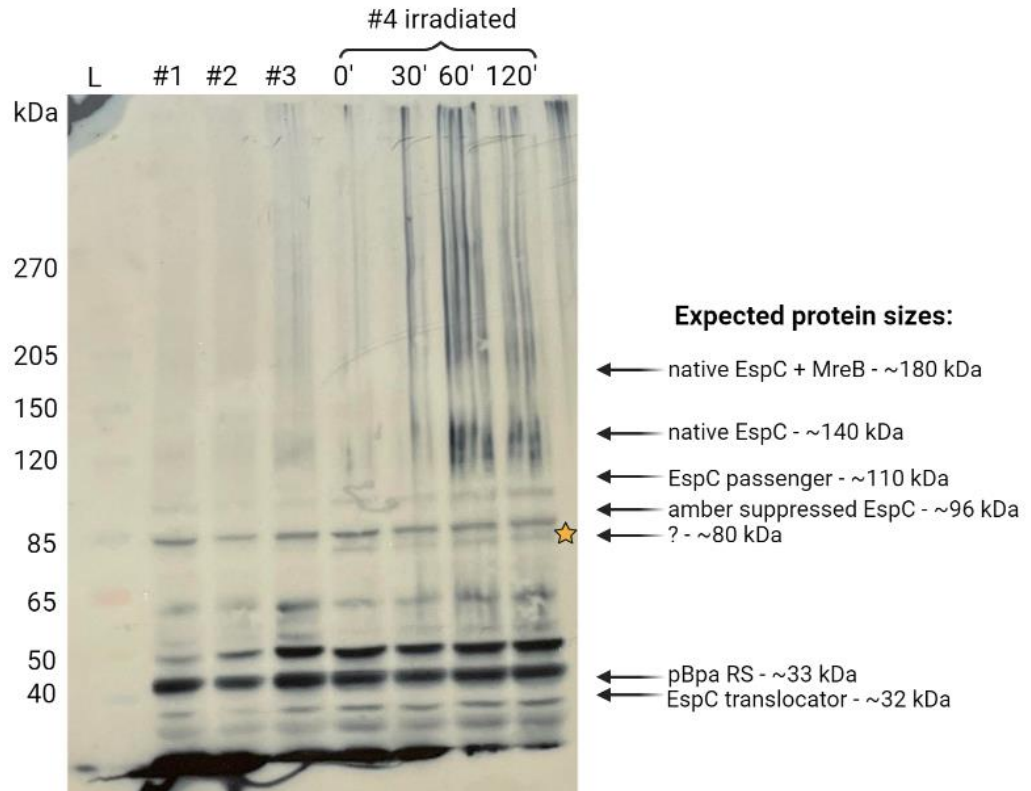


Figure 4.13. Crosslinking of EspC via pBpa did not yield distinct protein complexes for further analysis. The migration pattern of expected (predicted) protein sizes is indicated with arrows on the right. A protein the size of ~80 kDa unique to photoactivated samples is indicated with the yellow star. L – ladder; #1 – EspC (pLA33C1); #2 – pLA33C1TAG951; #3 pLA33C1TAG951 + pBpaRS – pBpa; #4 pLA33C1TAG951 + pBpaRS + pBpa; 0-120' = photo irradiation times in min.

4.3. Discussion

ATs were considered self-sufficient for a long time, encoding everything they need to be secreted. New research, however, showed the many gaps in understanding these proteins and how they interact with neighbouring molecules. For example, they require chaperones in the periplasm, and their OM translocation steps are still more theoretical than experimentally proven. This is why this chapter aimed to characterise EspC interactions on their way out of the cell, leading on from the observation it localises into a patchy helical structure reminiscent of helices produced by MreB and the Sec translocon (206).

Ashawesh *et al.* (2018) showed that when observing EspC in a SecA *ts* mutant, instead of the patchy, helical distribution, EspC aggregates at the poles of the cells, indicating it requires SecA for its normal localisation. It was necessary to test if a defect in any other major Sec components would result in a similar EspC displacement. Four *ts* mutants SecA_{ts}, SecY_{ts}, SecD_{ts} and SecF_{ts}, were cultured in parallel to the wild-type MG1655 *E. coli* to discount the possibility the unique effect on EspC localisation is the product of stress response induced by temperature fluctuation needed to activate the mutations. Indeed, although EspC kept its patchy localisation in wild-type *E. coli* cells at sub-optimal temperatures (42 °C, 25 °C), it was localised to the cell poles in the SecA and SecY *ts* mutants (Fig. 4.2., 4.3.). Considering that the Sec translocon is responsible for IM translocation of most proteins, it could be that native secretion/functionality of yet unidentified EspC interaction partners is attenuated in the *ts* mutants. However, the same mutants were previously used to probe the localisation of the AT IcsA of *S. flexneri*, where they found no differences between the wild-type and *ts* mutants, strengthening the argument these mutants are suitable for the study of protein localisation (115). There was no observable EspC-mCherry production in SecD_{ts} and SecF_{ts}. Since SecD and SecF form a heterodimer which uses the proton gradient to facilitate pre-protein IM crossing from the periplasmic side, their dysfunction would potentially cause the SecYEG channel to be blocked by the pre-proteins (270). Although these *ts*

mutants are not fatal (215), it would be prudent to check for cell viability using a dye (e.g. live/dead bacterial staining) to eliminate the possibility of cell death caused by culturing and mounting methods.

It was next decided to study the interaction of EspC (EspC-mCherry, pLA33C1mc) and the helical actin homologue MreB (MreB-TC, pLA33mreb-TC) using the existing constructs prepared by Ashawesh (207). It was essential to establish whether the constructs were fit for purpose and accurately portrayed real-life interactions. In all microscopy experiments, only 0.02% arabinose (usually 0.2%) was used as the inducer to prevent excess recombinant protein production that could cause clustering artefacts (207). Although EspC localisation hasn't previously been studied, multiple studies were done to discern the nature of MreB filaments. Dominguez-Escobar *et al.* (2011) found that MreB forms 'discrete patches' which, when imaged axially, look like long helical filaments (254). On the other hand, van Teeffelen *et al.* (2011) found MreB to display the 'spotty' localisation but also reported short diagonal bands, which they thought more accurately represented the protofilaments (172). Relatively recent imaging using SR microscopy in *B. subtilis* revived the model for MreB forming extended filaments along the cell (271, 272) and the discrepancies in these findings (and the ongoing controversy) are attributed to weighty FPs used in previous attempts, the shortcomings of conventional confocal microscopy and imaging conditions which could impact cell shape (e.g. use of coverslips) (273). The findings reported here support a helical distribution of MreB, strengthened by the use of FIASH technology instead of FPs, and using SR-SIM (207).

MreB and EspC colocalise as discrete patches along the length of *E. coli* MG1655 cells that successfully produce both plasmids (Fig. 4.4-6.). The effect of differential recombinant protein production is probably seen because the FIASH labelling is not saturated, resulting in MreB-TC that is not fluorescent plus the endogenous unlabelled MreB, which the cells preferentially integrate (Fig. 4.5.). On closer inspection and using 3D SR-SIM, the patches were observed further, revealing that, while the two

proteins colocalise, there are small areas of where the two fluorophore signals don't fully overlap. No 3D colocalisation analyses could be performed because the cells were collapsed (flattened) when imaged on 1.2% agarose pads (Fig. 4.7.). While the average width of *E. coli* MG1655 cells containing EspC-TC or MreB-TC is $\sim 0.7 \mu\text{m}$, the average width of cells containing EspC-mCherry and MreB-TC is closer to $0.4 \mu\text{m}$ (see below) (207). The small areas of discrepancy between the signals led to the speculation that SR-SIM provides a believable snapshot of real-life PPIs (no 'perfect' overlap due to their dynamic nature). Several verification methods were used to confirm colocalisation.

Pearson's correlation coefficient (PCC) takes into account co-occurrence (spatial overlap of two probes) and correlation (overlap and codistribution around the cell) of two fluorophore signals by measuring pixel-to-pixel covariance regardless of signal intensity (274). With a strong value of $\text{PCC} = 0.968$ (of maximum 1.0), EspC-mCherry and MreB-TC clearly colocalise. This score also indicates the two proteins are likely located in the same cellular compartment (274). Due to the FIAsh-EDT₂ reliance on reducing environments (the cytoplasm), it's likely that the two proteins are closely associated at the IM (229). The PCC value was backed up by Costes' randomization bioanalysis (also available as a part of the JACoP ImageJ plugin, see section 2.14.), which yielded $P = 100\%$, meaning no randomised images of EspC-mCherry ($n = 10000$) provided a similar correlation to MreB-TC than the one acquired by SR-SIM, strengthening the evidence of co-occurrence and potential interaction (275). Moreover, using Costes' automatic thresholding eliminated human bias from determining the optimal signal-noise ratio upon which to base colocalisation experiments. In the future, colocalisation should be investigated in Ashawesh's truncated EspC construct, which lacks the translocator he found essential for the patchy EspC localisation (207). Disturbing EspC localisation should provide a scenario where MreB and EspC do not colocalise, fortifying the evidence presented above.

As mentioned previously, it was not possible to perform 3D colocalisation analyses of the cells due to flattening, which occurred in all samples. The

flattening was confirmed when Tetraspeck fluorescent microspheres (Invitrogen, see section 2.15.1.) were added to mounted samples to calibrate microscope settings and prove the flattening is not an artefact. It was therefore decided to find alternative ways of mounting the samples. These had to have similar optical and fixative properties of agarose pads to match the quality of so far obtained *in vivo* images. The precision and super-resolution of SR-SIM come from the variety of grating angles (rotations) used during image acquisition (the image with the first rotation is taken, the grid is rotated, and the sample is scanned again, etc.). The photo-bleaching resulting from prolonged fluorophore excitation must be minimised to leave enough signal intensity for all five rotations. SR-SIM algorithms working with too little information will cause mathematical artefacts (spotted easily by their overly geometric patterns caused by the gratings). Although the use of CFM3 helped the photostability of mCherry (Fig. 4.8.A.), it did not adhere cells to the coverslip, making them unsuitable for imaging due to the length of image acquisition. Next, Poly-D-Lysine (synthetic extracellular matrix, Sigma) was added as a fixative to CFM3 mounted cells. Unfortunately, the addition of Poly-D-Lysine resulted in expedited photo-bleaching of the usually robust mCherry signal, dropping to almost half its intensity after the first rotation (4.8.A-B). The collapsed cell morphology occurs when cells are imaged *in vitro*, too (Fig. 4.10.). It most likely occurs as the result of the incorporation of the sizeable mCherry FP (~27 kDa). Alternatively, since the study focused on the possibility of MreB-mediated protein targeting of EspC, the size of EspC-mCherry bound to MreB filaments could cause the cell to collapse. There was no reference to cell flattening in Ashawesh's thesis (2016), but both EspC-TC and MreB-TC constructs he used showed no signs of flattening (206). The collapsed morphology could also be probed in 3D cells containing only one plasmid at a time (see Fig. 4.5.), which were presently excluded from colocalisation analyses.

When treated with A22, *E. coli* MG1655 circularises in response to MreB polymerisation inhibition (Fig. 4.9., 4.11.). It was hypothesised that EspC would diffuse around the circular cell, providing additional insight into the

relationship between EspC secretion and a functioning cytoskeleton. Although there was EspC diffused in the centre of the cell, surprisingly, some EspC still colocalised with MreB at the IM (Fig. 4.10.). EspC secretion was likely impeded, but whether that was an indirect consequence of A22 inhibition or directly related to the inability of MreB to self-polymerise cannot be deduced from fluorescent microscopy alone. Furthermore, the flat cells remained a problem for 3D analyses even when fixed with 0.1% glutaraldehyde (Fig. 4.10.). However, imaging the cells *in vitro* noticeably enhanced the signal intensity of MreB-TC.

Using pBpa to photo-crosslink EspC to neighbouring molecules, up to 4 Å away, is the next step to confirm EspC interactions during secretion. Due to time limitations, it wasn't possible to fully optimise the protocol for working with EspC constructs. Although it was possible to successfully resolve most of the crosslinked samples and control samples using Western blot with α -EspC, there were too many non-specific bands to identify proteins (Fig. 4.13.). No crosslinked samples (Fig. 4.13.) resolved proteins at ~180 kDa (roughly the combined molecular weight of MreB and EspC). In fact, no crosslinked complexes above 100 kDa could be resolved or identified. This persisted throughout changing the sample loading volume (from recommended 25 μ L to 5 μ L), the addition of a sonication step, using commercial gels designed for resolving high molecular weight proteins and slow electrophoresis times (see section 2.11.). Given how toxic UV light is to cells and proteins, it was also decided to use Pyrex glass Petri dishes to limit UV illumination to wavelengths >300 nm and thus protect crosslinking samples from the more harmful rays and limit cellular debris in the samples (269). Due to time constraints and the delay of α -MreB antibody delivery, it was not possible to immunoprecipitate crosslinked EspC (using α -EspC antibody) and follow it up with a Western blot using α -MreB. Other crosslinked partners could then be identified using mass spectrometry (267, 268).

Although Ashawesh's fractionation experiments show EspC-mCherry efficiently reaches the OM without forming aggregates, it is currently unclear if EspC-mCherry is imaged on the periplasmic or the cytoplasmic

side of the IM due to the fact mCherry effectively folds in both compartments (207, 225). It is therefore essential to fully optimise pBpa crosslinking to rule out one compartment or the other by showing a direct interaction between EspC and MreB. Alternatively, no interactions found between EspC and the periplasmic components of the elongasome (e.g. RodA, RodZ, MreC) would accomplish the same (276).

In this chapter, it has been demonstrated that the use of mCherry in combination with FIAsh technology is a good approach to visualise secreted proteins and their predicted interaction partners before they exit the cell. The two fluorophores both produce strong and mutually independent signals that are suitable for 2D *in vivo* colocalisation experiments. The colocalisation of EspC-mCherry and MreB-TC contributes to the emerging evidence that PG synthesis (which is coordinated by MreB) also serves as a system to target membrane proteins to their cellular destinations (174, 175, 176, 177, 178). However, this is the first time the colocalisation of MreB with a secreted protein was shown. The partial colocalisation of EspC-mCherry and MreB-TC in A22-treated cells also hints at the importance of MreB dynamics for EspC secretion. How these findings fit into the broader picture of 'classical' AT secretion is discussed in Chapter 6.

5. Insights into the inhibition of EspC secretion using a T3SS inhibitor Aurodox

5.1. Introduction

Bacteria have evolved regulatory molecules which modulate gene expression on a transcriptional level to synthesise proteins that best fit their needs in response to internal and external stimuli. Virulence genes are expressed after bacteria initiate pathogenesis, so they often fall under the umbrella of a single transcriptional regulator. In EPEC and EHEC, most virulence factors are encoded in LEE, a PAI consisting of five operons *LEE1-5* (9). Due to the reported differences between EHEC and EPEC virulence, this short review will only focus on EPEC (277). The entire T3SS is encoded on *LEE1-4*, while *LEE5* encodes Tir and intimin, crucial for forming A/E lesions (see section 1.2., (278)). The LEE1-encoded master regulator Ler is the transcriptional activator of EPEC virulence genes (both LEE-encoded and non-LEE encoded) (278, 279, 280). Ler's mode of action is counteracting the activity of the global silencer of Gram-negative bacteria, H-NS, by competing for DNA binding sites (281, 282, 283). Since then, many more regulatory factors and environmental signals were found to contribute to LEE expression (284). Ler has also been shown to auto-regulate (285). The expression of *ler* involves a regulatory cascade of IHF (integration host factor), PerC and GrlA (global regulator of LEE-activator; LEE-encoded) to overcome H-NS silencing (Fig. 5.1.) (282, 286). The production of many of these regulatory enzymes was proven to be stimulated by changes in temperature and switches from high- to low-glucose media (280, 287). As mentioned above, Ler activates non-LEE-encoded virulence genes, including the EspC AT (288). Moreover, the interplay between T3 and T5SS is required for successful EspC host cell internalisation (243).

Aurodox was first isolated and purified from *S. goldiniensis* cultures in 1972 after Berger *et al.* noticed its antimicrobial activity during a screen for new antibiotics (289). Although its *in vitro* performance outshined that of the closely related compound lincomycin (both are kirromycins) from

Streptomyces lincolnensis, the very narrow spectrum of efficacy *in vivo* (good activity only against *S. pyogenes* in mice) halted the development of Aurodox as a commercial antibiotic (289).

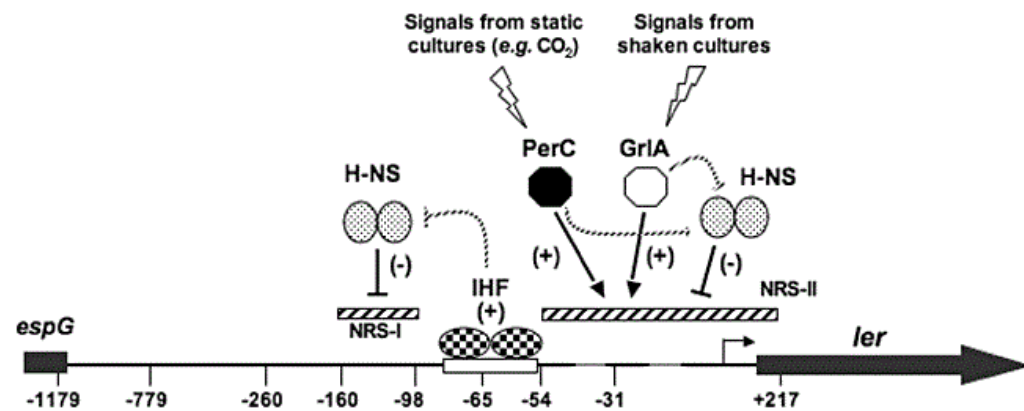


Figure 5.1. An overview of the *ler* transcriptional regulatory cascade. Before pathogenesis is initiated, EPEC and EHEC virulence genes are silenced by H-NS which binds DNA at two sites upstream of the *ler* promoter. The silencing is reversed upon the binding of IHF to the negative regulation site I (NRS-I). This activates *ler* transcription and allows the modulation of virulence gene expression by PerC and GrlA which are able to bind to the other site (NRS-II). PerC and GrlA modulate *ler* expression in response to environmental conditions. *In vitro*, this occurs when culturing the strains in DMEM at 37° C. Finally, Ler functions as a transcriptional activator of LEE and non-LEE encoded virulence genes, including some secreted by the T5SS (e.g. EspC). This figure was adapted from Bustamante *et al.* (282).

Decades later, Aurodox was re-discovered by Kimura *et al.* (2009) as a potential antivirulent (also known as anti-infectant) for the T3SS. Apart from observing the inability of cells treated with Aurodox to demonstrate T3SS-mediated haemolysis *in vitro*, they also showed that all mice infected with *Citrobacter rodentium* and treated with Aurodox survived the infection (as opposed to no survival in control groups) (204). Taking into account how essential & abundant EF-Tu is and the mode of action of kirromycins (see section 1.8.5.), it was unclear why Aurodox was found to inhibit EPEC virulence without impacting cell growth. A recent study has characterised a new gene (*aurM*) which codes for a SAM-dependent methyltransferase that methylates the pyridone ring of kirromycin as a final step in Aurodox biosynthesis (201). This ‘magic methyl effect’ is commonly exploited to increase the potency of biomolecules (290). McHugh *et al.* (2019) narrowed down the source of the inhibitory activity of Aurodox to a decrease in gene expression of *ler* and Ler-dependent genes with validated RNA sequencing data and the capability to restore

virulence in strains overexpressing *ler* (205). Notably, there was no change in the expression of EHEC SOS genes, indicating that Aurodox treatment causes no detectable stress response.

Here, the effect of Aurodox was investigated on the secretion of EspC; a non-LEE encoded AT of EPEC E2348/69, regulated by Ler (288). Reverse transcription-quantitative PCR was performed to confirm EspC secretion inhibition happens on a transcriptional level to substantiate the findings of McHugh *et al.* and Kimura *et al.* (204, 205).

5.2. Results

5.2.1. The T3SS inhibitor Aurodox inhibits EspC secretion

As explained above, Kimura *et al.* noted Aurodox's inhibitory activity on the T3SS and investigated its effect on LEE-encoded effectors EspB (T3SS translocase) and EspF (disrupts host cell tight junctions) (204). Using supernatant protein secretion profiles generated by SDS-PAGE of EPEC E2348/69 cultures treated with increasing concentrations of Aurodox, they also reported the decrease in the secretion of a ~110 kDa protein they identified as EspC. Here, a concentration-dependant reduction in EspC secretion was confirmed when treated with 0 (control), 1.25, 2.5, 3.75 and 5 µg/mL Aurodox. All concentrations were checked for growth impediments (OD_{600nm} measured after the 5-hour incubation, data not shown). Secreted proteins were analysed as described in 2.9. and Western blotting was performed using the α-EspC polyclonal antibody generated by Keith Bishop (see section 2.8.3.) (221). Three biological replicates were tested, and the amount of secreted protein was calculated according to protein band intensity (relative quantification, RQ) using ImageJ. The size of EspC was confirmed by referencing the SDS-PAGE ladder, and the results have been compiled into a graph (Fig 5.2.). The bar graph shows a steady decrease in EspC transcription when treated with increasing concentrations of Aurodox. The results were also verified using Western blotting with the α-EspC antibody. Statistical analysis of the secretion inhibition was performed using a repeated measures one-way ANOVA. The ANOVA results showed Aurodox significantly decreases EspC secretion ($p = 0.0176$) and confirmed the effective matching of values between the biological replicates ($p = 0.0436$).

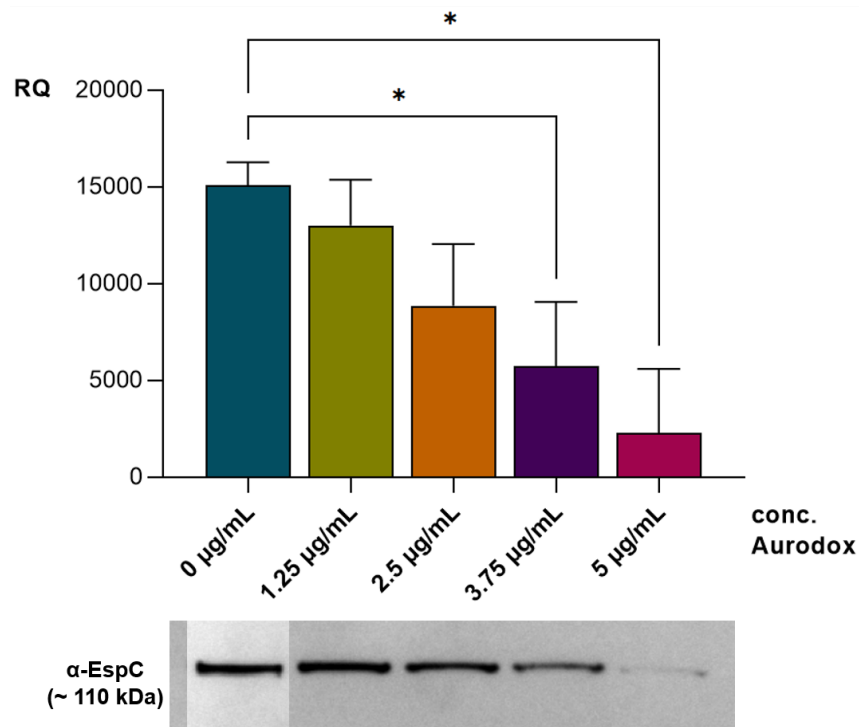


Figure 5.2. EspC secretion decreases when treated with the T3SS inhibitor Aurodox. Kimura *et al.* reported the decrease in the secretion of a ~110 kDa protein in EPEC when investigating the effect of Aurodox on secretion of EspB and EspF. Here the use of a specific polyclonal antibody and Western blotting confirms a reduction in EspC in the presence of increasing concentrations of Aurodox. Statistical significance (*) between treatment groups was found when comparing the control (0 µg/mL) to samples treated with 3.75 µg/mL ($p = 0.0374$) and 5 µg/mL Aurodox ($p = 0.0329$). RQ – relative quantification; conc. – concentration; α-EspC – anti-EspC polyclonal antibody.

5.2.2. EspC inhibition by Aurodox occurs at the transcriptional level

McHugh *et al.* (205) revealed Aurodox inhibits the expression of *ler*. To confirm this, the gene expression of *espC* was assessed, using RT-qPCR as described in 2.10., in the presence of Aurodox. To determine the optimal concentration of primers for the qPCR reaction, primer pair rtMS1-2 was tested for their cDNA amplification ability using a range of 50-900 nM final primer concentration (rtMS1 conc. – rtMS2 conc., respectively) (Fig 5.1.). Published primers were selected, and the 16S ribosomal RNA gene *rrnB* was used as the endogenous control (185, 186).

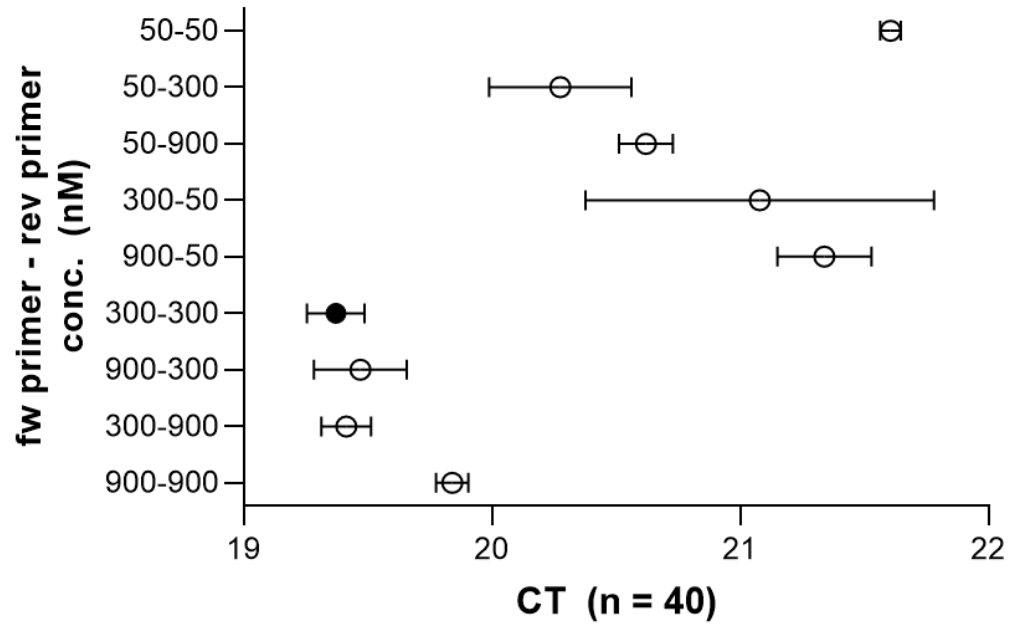


Figure 5.4. Final concentrations of *espC* qPCR primers were determined according to their amplification of EPEC gDNA. A range of different qPCR primer concentrations were tested for their ability to amplify 1 μ L of EPEC E2348/69 genomic DNA, as described in 2.10. The first and second numbers denote the final concentrations of rtMS1 (*espC* forward) and rtMS2 (*espC* reverse). The 300 nM concentration was chosen for both primers (black circle) for the use in gene expression experiments based on its early detection of gDNA in cycle 19 (out of 40 in total). CT – cycle threshold.

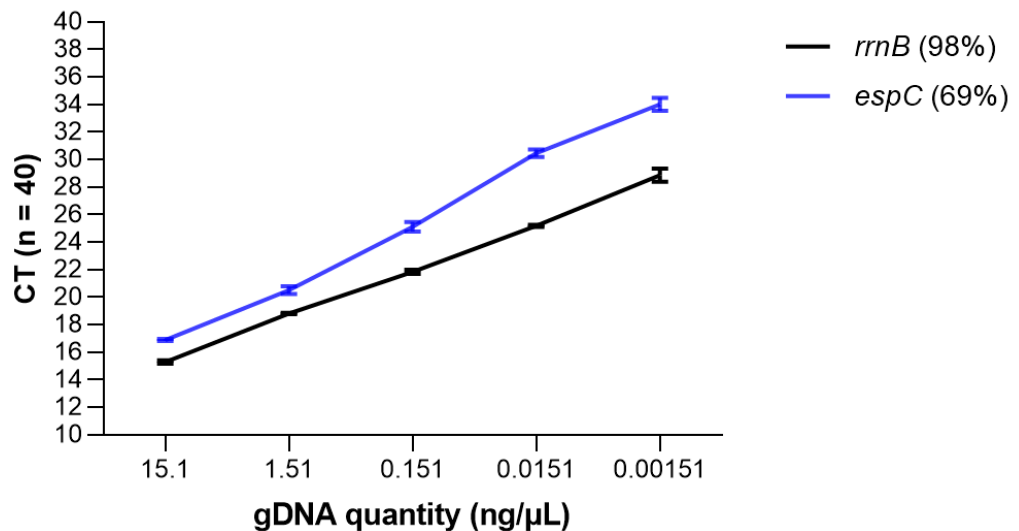


Figure 5.3. Standard amplification curves were created for *espC* and *rrnB* using EPEC genomic DNA. Standard curves were created for primer pairs rtMS1-2 (*espC*) and rtMS3-4 (*rrnB*). The already published *espC* primers showed only 69% efficiency of EPEC gDNA amplification, as calculated by the Applied Biosystems RT-PCR software. The endogenous control showed near perfect efficiency. CT – cycle threshold; gDNA – genomic DNA).

Both primer pairs were used at the final concentration of 300 nM. Standard curves were obtained for *espC* (69% primer efficiency) and *rrnB* (98% primer efficiency) by testing their amplification of EPEC gDNA (Fig. 5.2.). The efficiency test was repeated for *espC* to exclude the possibility of manual pipetting errors. Still, the same result was obtained (Fig. 5.2.). The PCR products' melting points (T_m) were examined. Only one peak appeared at the expected temperature ($\sim 77^\circ\text{C}$), indicating its low efficiency is unlikely to be due to primer non-specificity.

Three biological replicates of EPEC E2348/69 were grown in 5 mL LB overnight at 37°C with shaking. The $\text{OD}_{600\text{nm}}$ of each replicate was normalised to 0.05, treated with 0, 2.5 or 5 $\mu\text{g/mL}$ Aurodox and incubated for an additional 5 h. RNA extraction was performed immediately, and RNA integrity and quality were analysed using the Agilent RNA 6000 Nano Kit according to manufacturer instructions (Fig 5.3., see section 2.10.).

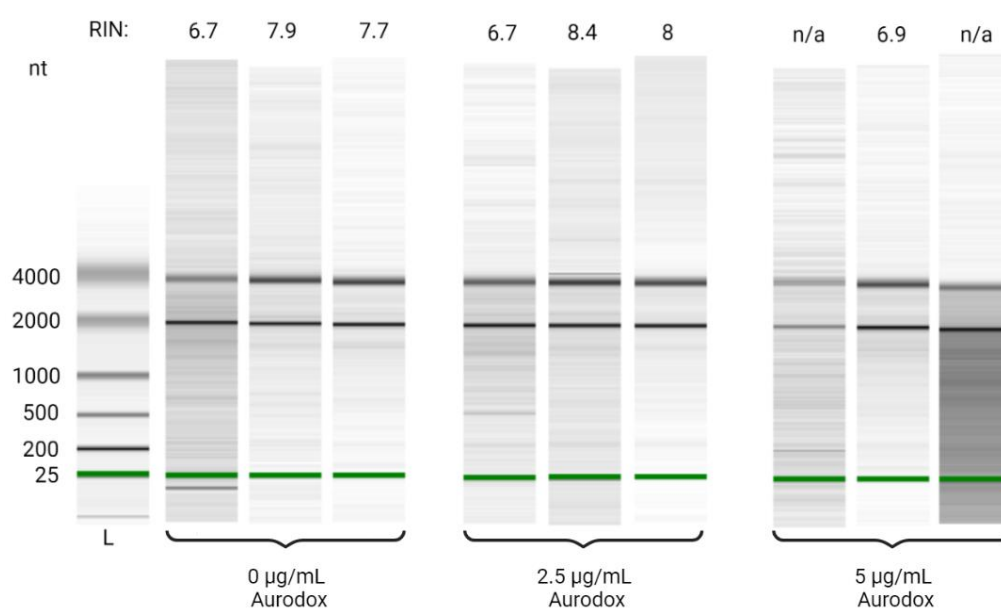


Figure 5.5. RNA degradation was observed for EPEC E2348 samples incubated with 5 $\mu\text{g/mL}$ Aurodox. The RNA integrity of EPEC E2348/69 samples treated with Aurodox for 5 h was measured using the Agilent 2100 Bioanalyzer. Following extraction, RNA samples were treated with the TURBO DNA-free™ kit (Invitrogen) to degrade residual DNA. Although most of the extracted RNA were of acceptable quality (RIN > 5), the RNA of samples treated with 5 $\mu\text{g/mL}$ were degraded to the point of not being able to assign them a RIN number. At this time, it is impossible to say if this is due to the effect of Aurodox, but both degraded samples showed no signs of contamination during downstream analysis. RIN – RNA Integrity Number (out of 10); L – ladder; nt – nucleotides; n/a – not applicable, degraded.

The reverse transcriptase step was performed to convert RNA into complementary DNA (cDNA). The purity of RNA was confirmed (19 amplification cycles difference before the first appearance of background DNA) using a series of cDNA dilutions and no template controls. A standard quantification curve was performed using the 7500 Real Time PCR System (Applied Biosystems™), the SYBR™ Green PCR Master Mix (Applied Biosystems™) and 0.1 µL cDNA. Amplification conditions are listed in Table 5.1. Two out of the three biological replicates of the 5 µg/mL group showed signs of DNA degradation, whereas other samples were of high (RIN ≥ 8) or acceptable quality (RIN > 5) (291). Nevertheless, when tested, the samples with no RIN score showed no abnormalities or signs of DNA contamination or non-specific amplification, so they were included in gene expression analyses. Gene expression analyses of the three treatment groups revealed a decrease in *espC* transcription in samples treated with Aurodox. At 5 µg/mL, the reduction was close to 2-fold (1.67-fold) to that of untreated EPEC E2348/69 (Fig 5.6-7).

Cycle stage	Temperature	Length
Initial denaturation	95 °C	10 min
	95 °C	15 sec
25 - 35 cycles	60 °C	30 sec
	72 °C	30 sec

Table 5.1. Amplification conditions for RT-qPCR. For the complete method, see section 2.10.

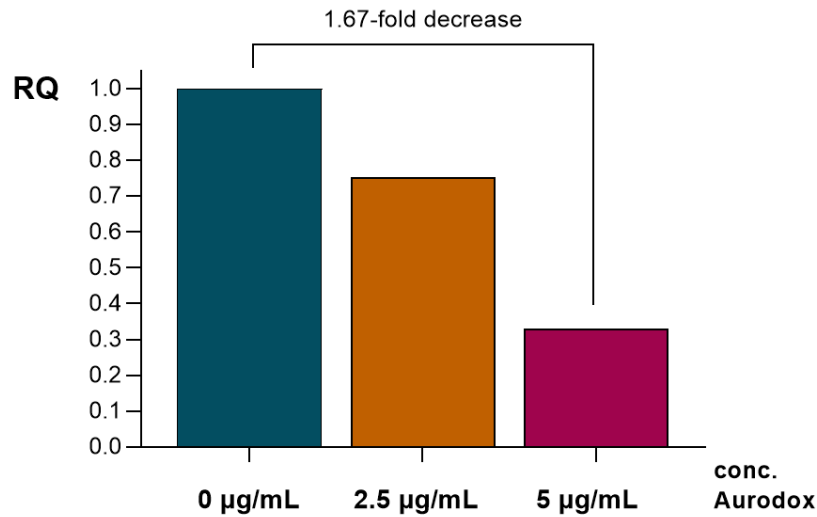


Figure 5.6. The results of relative quantifications of gene expression show a 1.67-fold decrease in *espC* transcription in samples treated with sub-bacteriostatic levels of the T3SS inhibitor Aurodox. This confirms the transcriptional deactivation of T3SS results in the indirect suppression of T5SS-related virulence caused by EPEC E2348/69 during IEC infection. RQ – relative quantification.

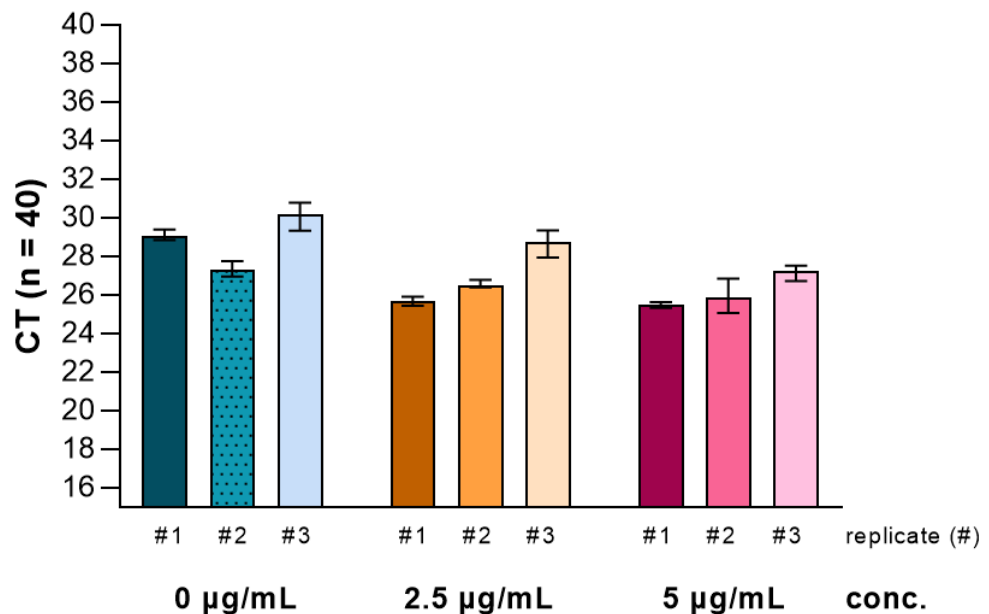


Figure 5.7. An overall decrease in *espC* mRNA qPCR detection is observed in samples which received Aurodox treatment. CT values for three biological replicates of EPEC E2348/69 treated with Aurodox are shown as 1 – blue, 2 – orange and 3 – magenta bars on the x axis. In all replicates *espC* mRNA is detected a couple of cycles before the treated groups, with the exception of the first replicate where a contaminant was detected in the 2.5 µg/mL group (likely a pipetting error during cDNA dilution). CT – cycle threshold; cDNA – complimentary DNA; conc. – concentration.

5.3. Discussion

Although reports on promising antivirulents are regularly published, only five drugs have been approved by the FDA (e.g. bezlotoxumab for *C. difficile*) (292). These are human monoclonal antibodies targeting specific toxins, making them expensive to produce and not recommended over conventional medication (292, 293). On the other hand, compounds like Aurodox are readily purified from *S. goldiniensis* cultures, making their manufacturing as cost-efficient as naturally-derived antibiotics (201). Moreover, the Aurodox T3SS inhibitory activity in EPEC and EHEC is achieved using only micrograms of the compound, while its bactericidal properties were previously tested using 1 mg/mL (289). Together, these make Aurodox an ideal candidate for inexpensively preventing LEE-mediated virulence caused by EPEC, EHEC, *C. rodentium* and *Escherichia albertii*, especially since it has already been proven to work in mice *in vivo* (204).

As a member of the elfamycin family of antibiotics, Aurodox should stop protein synthesis by preventing EF-Tu dissociation from the ribosome, killing the cell shortly after (see section 1.8.5.). It was unknown how Aurodox was able to show inhibitory activity against the T3SS with its structure almost entirely identical to kirromycin, but the answer lies in a single methylation of kirromycin's pyridone ring, facilitated by the newly identified methyltransferase *aurM* present in Aurodox's biosynthesis gene cluster (see section 5.1.). McHugh *et al.* (2018) have narrowed the cause of Aurodox's inhibitory activity to a decrease in the expression of the master regulator Ler, indicating Aurodox either directly competes for its binding sites or affects an upstream regulator of *ler* (205, 284). Of ~80 EHEC chromosomal genes downregulated following Aurodox treatment, *grlA*, *ler*, *rpoE* and *sepL* belong to those previously found to be involved in LEE transcriptional regulation in EPEC (205, 284). GrlA and SepL require activation by Ler, so it is unsurprising to see all three downregulated in the presence of Aurodox (286, 294). Interestingly, RpoE is known to indirectly repress Ler in response to zinc, causing the same inhibition of LEE-mediated virulence as Aurodox-treated

EPEC/EHEC (including inhibition of non-LEE factors like EspC) (295). Since RpoE was found to be downregulated by McHugh in the presence of Aurodox, it's unlikely the two inhibitory effects are related.

It was decided to investigate Aurodox's effect on EspC. This Ler-regulated, non-LEE-encoded secreted protein utilises the T5SS to exit the cell and was shown to mediate T3SS pore formation at the host cell membrane (73) when treated with Aurodox, but here its correct identification was confirmed using Western blots with the α -EspC antibody (Fig. 5.2.). By investigating a non-LEE-encoded protein which utilises a separate secretion machinery to that of T3SS, it was necessary to substantiate McHugh's findings that the Aurodox mode of action is on *ler* directly (205). It was confirmed that *espC* gene expression is reduced 1.67-fold when treated with 5 μ g/mL Aurodox (Fig. 5.6.). This concurs with a decrease in gene expression due to the transcriptional inactivation of Ler, which would prevent Ler from activating the transcription of eEspC.

Several compounds exhibiting the same effect on virulence gene expression as Aurodox have been identified, but their exact mechanism of inhibiting transcriptional regulators has not yet been characterised (296, 297, 298, 299). It would be interesting to investigate whether Aurodox affects horizontally acquired regulators of LEE like Pch (Per in EPEC) since another antivirulent compound showed selectivity for both Pch and Ler (300). Additionally, a complete transcriptomic analysis of EPEC genes downregulated by Aurodox (using RNA sequencing) would identify potential inhibition targets upstream of *ler*.

6. Conclusions and future directions

This study was undertaken to develop new methods to further our understanding of the Type 5 AT secretion pathway (T5SS). ATs are often overlooked as major factors of bacterial virulence, but they are involved in crucial steps necessary for establishing infection - surface adhesion, motility, evasion of host immune responses, cytotoxicity, cytolysis etc. (29). They are widely spread amongst Gram-negative bacteria and have been 'translocating under the radar' for a long time due to their deceptively simple structure. Since they encode an IM-targeting moiety (the signal peptide), the functional 'passenger' and a C-terminal β -barrel essential for OM translocation, decades have passed thinking ATs can independently secrete themselves across the cell membranes. Since it was revealed that ATs require periplasmic chaperones and the BAM complex for their successful secretion, there has been renewed interest in characterising T5SSs (43). Since all ATs are either OM surface-tethered or released from the surface after OM translocation, they pose a convenient target for the new generation of antimicrobials called antivirulents which target virulence factors, circumventing the existing antimicrobial resistance mechanisms and preventing the emergence of new ones (see section 1.8.5.).

The problems AT researchers face when trying to obtain a complete picture of 'classical' AT secretion (T5a) have been outlined in Chapter 1. The field is filled with contradictory findings and ambiguous vocabulary, which increases the likelihood of drawing false parallels (see section 1.4.). It should be noted even closely related ATs can differ in the choice of IM-targeting pathway, chaperone interactions & dependencies, and differential utilisation of the OM machinery (BAM/TAM). One such family are serine protease ATs of Enterobacteriaceae (SPATEs) which, although consistently grouped together by phylogenetic analyses, don't seem to share a common secretion mechanism (see sections 1.4., 1.5.). This is most likely due to the diverse number of species they appear in, which are similar but distinct due to generations of speciation events. Phylogenetic analyses also find this to be true for ATs. Although

horizontally acquired long ago, they have since developed unique characteristics and likely 'perfected' their secretion using the available cellular machinery (83, 84).

Here, we have investigated the secretion mechanism of EspC, a SPATE from EPEC that causes cytotoxicity of intestinal epithelial cells (IECs) by cleaving a range of host factors (fodrin, spectrin etc.). EspC is also the first AT detected during EPEC infection of IECs, owing its timely expression to LEE-mediated virulence genes with which it shares transcriptional regulation by Ler. By use of fluorescent microscopy, photo-crosslinking and secretion machinery mutants, this study aimed to develop a reliable method to track EspC secretion and distinguish key proteins involved. This would help identify targets for the development of antivirulents, provide researchers with an approach to track ATs intracellularly and give insight into the ATs of related pathogens. The data presented in this study followed previous observations of intracellular EspC localising into discrete helical patches along the cells of *E. coli* MG1655 using mCherry and FIAsh-EDT₂ fusion proteins. Owing to the unusual nature of the localisation pattern (most proteins localise to the cell poles), concerns were raised about self-aggregation of the FP and the harshness of FIAsh-labelling potentially causing clustering artefacts. In Chapter 3, the focus was to develop a way of extracellularly labelling EspC-mCherry using SpyCatcher technology with hopes of proving unimpeded secretion of the FP fusions and a way to quantify secretion of EspC at the OM. Although native EspC was successfully tagged with SpyTag, no approach yielded an EspC-mCherry-ST mutant to compare it with (Fig. 3.5.B.). Nevertheless, EspC-ST can be used to visualise EspC upon entering mammalian cells. EspC is thought to utilise the T3SS to translocate from the extracellular milieu to host cells, but the mechanism is not understood. A novel role for EspC was recently found, regulating the levels of T3SS components EspA and EspD, suggesting EspC is important in modulating EPEC infection (the same was found for the EspC EHEC homologue EspP) (73).

Although it wasn't possible to validate the use of EspC-mCherry for protein localisation using methods outlined in Chapter 3, previous findings strongly indicate that patchy helical localisation is an accurate visualisation of intracellular EspC. Ashawesh demonstrated EspC-mCherry is detectable in culture supernatants and that no EspC-mCherry aggregates accumulate inside the cell using sucrose fractionation (207). It was therefore decided to continue with its use and test it in a range of Sec temperature-sensitive (ts) mutants (Chapter 4). EspC localisation was affected in both SecA_{ts} and SecY_{ts} mutants where it localised to the cell poles (Fig. 4.2.). No EspC-mCherry was detected in SecD_{ts} and SecF_{ts} (Fig. 4.3.). A recent study has demonstrated a direct interaction between the BAM complex and SecDF (but not SecYEG) as a part of the membrane-spanning super-complex (holo-translocon). It's hypothesised that the holo-translocon allows for the efficient secretion of OMPs, with enough room to accommodate periplasmic chaperones (301). Taking into account the increasing likeness of AT biogenesis to that of OMPs, the non-functioning holo-translocon could be the reason why SecD-F ts mutants caused the attenuation of EspC secretion.

In Chapter 4, the colocalisation of MreB and EspC was demonstrated using EspC-mCherry and MreB-TC, strengthening the evidence that the cytoskeletal shape-determining protein MreB has a role in targeting proteins to the cell membranes. It is unlikely that MreB-assisted targeting is used by all proteins, as demonstrated by the mislocalisation of the IcsA AT in cells treated with A22, whereas another polar protein CheY did not share the same fate (191). While researching peptidoglycan (PG) synthesis, Mamou *et al.* (2022) shed new light on how the proposed targeting works by showing stronger colocalisation of OMPs with PG than with the BAM complex they require for OM insertion. Moreover, they found BAM is localised uniformly at the OM, but its insertase activity there is inhibited by mature, tetrapeptide-rich PG, which is absent from the cell poles. The nascent PG (pentapeptide-rich) inserted at the poles does not share the same inhibitory activity (302). This fits in with what is currently

known about AT secretion and what was observed here when tracking EspC intracellularly.

Combining the conclusions inferred from the extensive literature research and experimental data reported here and by Ashawesh (206, 207), the following steps are proposed for EspC secretion:

- After protein translation, the pre-protein is recognised and bound by SecB to target EspC for Sec-assisted IM translocation (see section 1.3.1.1.)
- Through an unknown mechanism, EspC associates with MreB and uses its helical protofilaments as tracks to reach the cell poles of *E. coli* (see Chapter 4)
- (Due to the effect of *ts* mutants on EspC localisation, reported reliance of MreB on the Sec translocon and the fact SecYEG & SecA have been found to distribute helically as well, Sec might also play a role in the cell pole delivery) (see section 4.2.1.) (211)
- Upon arrival at the cell poles, EspC translocates across the SecYEG IM channel, assisted by SecA at the IM side and SecDF at the periplasmic side
- SecDF and BAM form a periplasm-spanning vestibule for the preparation of ATs by chaperones and the directed insertion into the OM by the BAM complex; the signal peptide is cleaved (301)
- BAM facilitates the insertion of the EspC translocator by recognising conserved motifs in the β -barrel, changing its conformation and incorporating EspC β -strands into its own OM channel, creating a 'hybrid barrel' intermediate which is big enough to allow OM EspC passenger translocation (156)
- The C-terminal part of the passenger diffuses through the intermediate barrel, and upon reaching the cell surface, it initiates sequential folding, which promotes the translocation of the rest of the passenger (158)
- The EspC β -barrel buds out of the BAM complex, and the conformational changes caused by passenger folding allow for the proteolytical cleavage to occur in the linker.

To this author's knowledge, no other extracellularly cleaved ATs have been investigated using fluorescent microscopy, making it currently impossible to compare the proposed early EspC secretion steps to that of other 'classical' ATs.

Although many elements in this study require further optimisation, the foundations were laid for new ways of investigating AT protein-protein interactions in *E. coli*. Direct interaction of EspC and MreB must be shown to substantiate the colocalisation experiments and the proposed targeting of EspC to the cell poles by the cytoskeleton, with a site-specific approach like pBpa photo-crosslinking being particularly suitable. When optimised, this crosslinking method could also be combined with pulse-chase to track EspC interactions through time, providing the much-needed details of AT secretion steps. EspC's involvement with the T3SS should be probed further, especially since they share a regulatory network. The obtained EspC-ST construct has the potential to be used to image EspC entering the host cell and its prolonged effect on IECs.

7. References

1. **WHO.** The top 10 causes of death 2020 [Available from: <https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death>].
2. **WHO.** Antimicrobial resistance: World Health Organization; 2021 [updated 17/11/2021. Available from: <https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance>].
3. **HSA.** *Escherichia coli* bacteraemia: annual data: National Statistics; **2014** [updated 15/09/2021. Available from: <https://www.gov.uk/government/statistics/escherichia-coli-e-coli-bacteraemia-annual-data>].
4. **WHO.** Diarrhoeal disease: Fact Sheet **2017** [Available from: <https://www.who.int/news-room/fact-sheets/detail/diarrhoeal-disease>].
5. **WHO.** Diarrhoea: World Health Organization; [Available from: https://www.who.int/health-topics/diarrhoea#tab=tab_1].
6. **Angulo-Zamudio U.A., Gutierrez-Jimenez J., Monroy-Higuera L., Flores-Villasenor H., Leon-Sicairos N., Velazquez-Roman J., et al.** Non-diarrheagenic and diarrheagenic *E. coli* carrying supplementary virulence genes (SVG) are associated with diarrhea in children from Mexico. *Microb Pathog.* **2021**;157:104994.
7. **Kotloff K.L., Nataro J.P., Blackwelder W.C., Nasrin D., Farag T.H., Panchalingam S., et al.** Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study. *Lancet.* **2013**;382(9888):209-22.

8. **Knutton S., Lloyd D.R., McNeish A.S.** Adhesion of enteropathogenic *Escherichia coli* to human intestinal enterocytes and cultured human intestinal mucosa. *Infect Immun.* **1987**;55(1):69-77.
9. **Croxen M.A, Law R.J., Scholz R., Keeney K.M., Wlodarska M., Finlay B.B.** Recent Advances in Understanding Enteric Pathogenic *Escherichia coli*. *Clinical Microbiology Reviews.* **2013**;26(4):822–80.
10. **Campellone K.G.** Cytoskeleton-modulating effectors of enteropathogenic and enterohaemorrhagic *Escherichia coli*: Tir, EspFU and actin pedestal assembly. *The Federation of European Biochemical Societies journal.* **2010**;277:2390-402.
11. **Phillips N., Hayward R.D., Koronakis V.** Phosphorylation of the enteropathogenic *E. coli* receptor by the Src-family kinase c-Fyn triggers actin pedestal formation. *Nature Cell Biology.* **2004**;6(7):618-25.
12. **Silva C., Crispín Z.A., Puente J.L.** Self-Conjugation of the Enteropathogenic *Escherichia coli* Adherence Factor Plasmid of Four Typical EPEC Isolates - ProQuest. *BioMed Research International.* **2017**;2017(6325736).
13. **Lacher D.W., Steinsland H., Blank E.T., Donnenberg M.S., Whittam T.S.** Molecular Evolution of Typical Enteropathogenic *Escherichia coli*: Clonal Analysis by Multilocus Sequence Typing and Virulence Gene Allelic Profiling. *Journal of Bacteriology.* **2007**;189(2):342.
14. **Hu J., Torres A.G.** Enteropathogenic *Escherichia coli*: foe or innocent bystander? *Clinical Microbiology and Infection.* **2015**;21(8):729-34.
15. **Sakkejsa H., Byrne L., Lawson A.J., Jenkins C.** An update on the microbiology and epidemiology of enteropathogenic *Escherichia coli*

in England 2010–2012. *Journal of Medical Microbiology*. **2013**;62:1531-4.

16. **Estrada-Garcia T., Lopez-Saucedo C., Thompson-Bonilla R., Abonce M., Lopez-Hernandez D., Santos J.I., et al.** Association of Diarrheagenic *Escherichia coli* Pathotypes with Infection and Diarrhea among Mexican Children and Association of Atypical Enteropathogenic *E. coli* with Acute Diarrhea. *Journal of Clinical Microbiology*. **2009**;47(1):93.

17. **Ochoa T.J., Barletta F., Contreras C., Mercado E.** New insights into the epidemiology of enteropathogenic *Escherichia coli* infection. *Trans R Soc Trop Med Hyg*. **2008**;102(9):852-6.

18. **Green E.R., Meccas J.** Bacterial Secretion Systems – An overview. *Microbiol Spectr*. **2016**;4(1).

19. **Dautin N.** Folding Control in the Path of Type 5 Secretion. *Toxins (Basel)*. **2021**;13(5).

20. **Loos M.S., Ramakrishnan R., Vranken W., Tsirigotaki A., Tsare E.P., Zorzini V., et al.** Structural Basis of the Subcellular Topology Landscape of *Escherichia coli*. *Front Microbiol*. **2019**;10:1670.

21. **Chatzi K.E., Sardis M.F., Economou A., Karamanou S.** SecA-mediated targeting and translocation of secretory proteins. *Biochim Biophys Acta*. **2014**;1843(8):1466-74.

22. **Sala A., Bordes P., Genevaux P.** Multitasking SecB chaperones in bacteria. *Front Microbiol*. **2014**;5:666.

23. **Jiang X., Ruiz T., Mintz K.P.** Characterization of the secretion pathway of the collagen adhesin EmaA of *Aggregatibacter actinomycetemcomitans*. *Mol Oral Microbiol*. **2012**;27(5):382-96.

24. **Christie P.J.** The Rich Tapestry of Bacterial Protein Translocation Systems. *Protein J.* **2019**;38(4):389-408.
25. **Lycklama a Nijeholt J.A., Driessen A.J.M.** The bacterial Sec-translocase: structure and mechanism. *Philosophical Transactions B R Soc Lond B Biol Sci.* **2012**;367(1592):1016-28.
26. **Dalbey R.E., Kuhn A., Zhu L., Kiefer D.** The membrane insertase YidC. *Biochim Biophys Acta.* **2014**;1843(8):1489-96.
27. **Smets D., Loos M.S., Karamanou S., Economou A.** Protein Transport Across the Bacterial Plasma Membrane by the Sec Pathway. *The Protein Journal.* **2019**;38(3):262-73.
28. **Frain K.M., Robinson C., van Dijl J.M.** Transport of Folded Proteins by the Tat System. *The Protein Journal.* **2019**;38(4):377-88.
29. **Meuskens I., Saragliadis A., Leo J.C., Linke D.** Type V Secretion Systems: An Overview of Passenger Domain Functions. *Front Microbiol.* **2019**;10:1163.
30. **Oberhettinger P., Leo J.C., Linke D., Autenrieth I.B., Schutz M.S.** The inverse autotransporter intimin exports its passenger domain via a hairpin intermediate. *J Biol Chem.* **2015**;290(3):1837-49.
31. **Jacob-Dubuisson F., Guerin J., Baelen S., Clantin B.** Two-partner secretion: as simple as it sounds? *Res Microbiol.* **2013**;164(6):583-95.
32. **Kiessling A.R., Malik A., Goldman A.** Recent advances in the understanding of trimeric autotransporter adhesins. *Med Microbiol Immunol.* **2020**;209(3):233-42.
33. **Goyal P., Krasteva P.V., Van Gerven N., Gubellini F., Van den Broeck I., Troupiotis-Tsailaki A., et al.** Structural and mechanistic

insights into the bacterial amyloid secretion channel CsgG. *Nature*. **2014**;516(7530):250-3.

34. **Bhoite S., van Gerven N., Chapman M.R., Remaut H.** Curli Biogenesis: Bacterial Amyloid Assembly by the Type VIII Secretion Pathway. *EcoSal Plus*. **2019**;8(2).

35. **Cianciotto N.P., White R.C.** Expanding Role of Type II Secretion in Bacterial Pathogenesis and Beyond. *Infect Immun*. **2017**;85(5).

36. **Gorasia D.G., Veith P.D., Reynolds E.C.** The Type IX Secretion System: Advances in Structure, Function and Organisation. *Microorganisms*. **2020**;8(8).

37. **Charro N., Mota L.J.** Approaches targeting the type III secretion system to treat or prevent bacterial infections. *Expert Opin Drug Discov*. **2015**;10(4):373-87.

38. **Wallden K., Rivera-Calzada A., Waksman G.** Type IV secretion systems: versatility and diversity in function. *Cell Microbiol*. **2010**;12(9):1203-12.

39. **Costa T.R.D., Harb L., Khara P., Zeng L., Hu B., Christie P.J.** Type IV secretion systems: Advances in structure, function, and activation. *Mol Microbiol*. **2021**;115(3):436-52.

40. **Chandran Darbar V., Waksman G.** Structural Biology of Bacterial Type IV Secretion Systems. *Annual Review of Biochemistry*. **2015**.

41. **Navarro-Garcia F., Ruiz-Perez F., Cataldi A., Larzabal M.** Type VI Secretion System in Pathogenic *Escherichia coli*: Structure, Role in Virulence, and Acquisition. *Front Microbiol*. **2019**;10:1965.

42. **Klauser T., Pohlner J., Meyer T.F.** Extracellular transport of cholera toxin B subunit using *Neisseria* IgA protease beta-domain: conformation-dependent outer membrane translocation. *EMBO J.* **1990**;9(6):1991-9.
43. **Ieva R., Bernstein H.D.** Interaction of an autotransporter passenger domain with BamA during its translocation across the bacterial outer membrane. *PNAS.* **2009**;106(45):19120-5.
44. **Drobnak I., Braselmann E., Chaney J.L., Leyton DL, Bernstein HD, Lithgow T, et al.** Of linkers and autochaperones: an unambiguous nomenclature to identify common and uncommon themes for autotransporter secretion. *Mol Microbiol.* **2015**;95(1):1-16.
45. **Yen M.R., Peabody C.R., Partovi S.M., Zhai Y., Tseng Y.H., Saier M.H.** Protein-translocating outer membrane porins of Gram-negative bacteria. *Biochim Biophys Acta.* **2002**;1562(1-2):6-31.
46. **Dautin N.** Serine Protease Autotransporters of Enterobacteriaceae (SPATEs): Biogenesis and Function. *Toxins.* **2010**;2:1179-206.
47. **Pokharel P., Habouria H., Bessaiah H., Dozois C.M.** Serine Protease Autotransporters of the Enterobacteriaceae (SPATEs): Out and About and Chopping It Up. *Microorganisms.* **2019**;7(12).
48. **Freire C.A., Santos A.C.M., Pignatari A.C., Silva R.M., Elias W.P.** Serine protease autotransporters of Enterobacteriaceae (SPATEs) are largely distributed among *Escherichia coli* isolated from the bloodstream. *Braz J Microbiol.* **2020**;51(2):447-54.
49. **Clarke K.R., Hor L., Pilapitiya A., Luirink J., Paxman J.J., Heras B.** Phylogenetic Classification and Functional Review of Autotransporters. *Front Immunol.* **2022**;13:921272.

50. **Habouria H., Pokharel P., Maris S., Garenaux A., Bessaiah H., Houle S., et al.** Three new serine-protease autotransporters of Enterobacteriaceae (SPATEs) from extra-intestinal pathogenic *Escherichia coli* and combined role of SPATEs for cytotoxicity and colonization of the mouse kidney. *Virulence*. **2019**;10(1):568-87.
51. **Patel S.K., Dotson J., Allen K.P., Fleckenstein J.M.** Identification and molecular characterization of EatA, an autotransporter protein of enterotoxigenic *Escherichia coli*. *Infect Immun*. **2004**;72(3):1786-94.
52. **Leyton D.L., Sloan J., Hill R.E., Doughty S., Hartland E.L.** Transfer region of pO113 from enterohemorrhagic *Escherichia coli*: similarity with R64 and identification of a novel plasmid-encoded autotransporter, EpeA. *Infect Immun*. **2003**;71(11):6307-19.
53. **Brunder W., Schmidt H., Karch H.** EspP, a novel extracellular serine protease of enterohaemorrhagic *Escherichia coli* O157:H7 cleaves human coagulation factor V. *Mol Microbiol*. **1997**;24(4):767-78.
54. **Djafari S., Ebel F., Deibel C., Kramer S., Hudel M., Chakraborty T.** Characterization of an exported protease from Shiga toxin-producing *Escherichia coli*. *Mol Microbiol*. **1997**;25(4):771-84.
55. **Otto B.R., van Dooren S.J., Nuijens J.H., Luirink J., Oudega B.** Characterization of a hemoglobin protease secreted by the pathogenic *Escherichia coli* strain EB1. *J Exp Med*. **1998**;188(6):1091-103.
56. **Eslava C., Navarro-Garcia F., Czeczulin J.R., Henderson I.R., Cravioto A., Nataro J.P.** Pet, an autotransporter enterotoxin from enteroaggregative *Escherichia coli*. *Infect Immun*. **1998**;66(7):3155-63.

57. **Benjelloun-Touimi Z., Sansonetti P.J., Parsot C.** SepA, the major extracellular protein of *Shigella flexneri*: autonomous secretion and involvement in tissue invasion. *Mol Microbiol.* **1995**;17(1):123-35.
58. **Dozois C.M., Dho-Moulin M., Bree A., Fairbrother J.M., Desautels C., Curtiss R. 3rd.** Relationship between the Tsh autotransporter and pathogenicity of avian *Escherichia coli* and localization and analysis of the Tsh genetic region. *Infect Immun.* **2000**;68(7):4145-54.
59. **Sandt C.H., Hill C.W.** Four different genes responsible for nonimmune immunoglobulin-binding activities within a single strain of *Escherichia coli*. *Infect Immun.* **2000**;68(4):2205-14.
60. **Gutierrez D., Pardo M., Montero D., Onate A., Farfan M.J., Ruiz-Perez F., et al.** TleA, a Tsh-like autotransporter identified in a human enterotoxigenic *Escherichia coli* strain. *Infect Immun.* **2015**;83(5):1893-903.
61. **Schmidt H., Zhang W.L., Hemmrich U., Jelacic S., Brunder W., Tarr P.I., et al.** Identification and characterization of a novel genomic island integrated at selC in locus of enterocyte effacement-negative, Shiga toxin-producing *Escherichia coli*. *Infect Immun.* **2001**;69(11):6863-73.
62. **Al-Hasani K., Rajakumar K., Bulach D., Robins-Browne R., Adler B., Sakellaris H.** Genetic organization of the *she* pathogenicity island in *Shigella flexneri* 2a. *Microb Pathog.* **2001**;30(1):1-8.
63. **Parreira V.R., Gyles C.L.** A novel pathogenicity island integrated adjacent to the thrW tRNA gene of avian pathogenic *Escherichia coli* encodes a vacuolating autotransporter toxin. *Infect Immun.* **2003**;71(9):5087-96.

64. **Gibold L., Garenaux E., Dalmasso G., Gallucci C., Cia D., Mottet-Auselo B., et al.** The Vat-AIEC protease promotes crossing of the intestinal mucus layer by Crohn's disease-associated *Escherichia coli*. *Cell Microbiol.* **2016**;18(5):617-31.
65. **Mellies J.L., Navarro-Garcia F., Okeke I., Frederickson J., Nataro J.P., Kaper J.B.** *espC* pathogenicity island of enteropathogenic *Escherichia coli* encodes an enterotoxin. *Infect Immun.* **2001**;69(1):315-24.
66. **Roy K., Kansal R., Bartels S.R., Hamilton D.J., Shaaban S., Fleckenstein J.M.** Adhesin degradation accelerates delivery of heat-labile toxin by enterotoxigenic *Escherichia coli*. *J Biol Chem.* **2011**;286(34):29771-9.
67. **Khan S., Mian H.S., Sandercock L.E., Chirgadze N.Y., Pai E.F.** Crystal structure of the passenger domain of the *Escherichia coli* autotransporter EspP. *J Mol Biol.* **2011**;413(5):985-1000.
68. **Otto B.R., Sijbrandi R., Luirink J., Oudega B., Heddle J.G., Mizutani K., et al.** Crystal structure of hemoglobin protease, a heme binding autotransporter protein from pathogenic *Escherichia coli*. *J Biol Chem.* **2005**;280(17):17339-45.
69. **Domingo Meza-Aguilar J., Fromme P., Torres-Larios A., Mendoza-Hernandez G., Hernandez-Chinas U., Arreguin-Espinosa de Los Monteros R.A., et al.** X-ray crystal structure of the passenger domain of plasmid encoded toxin(Pet), an autotransporter enterotoxin from enteroaggregative *Escherichia coli* (EAEC). *Biochem Biophys Res Commun.* **2014**;445(2):439-44.
70. **Kenny B., Finlay B.B.** Protein secretion by enteropathogenic *Escherichia coli* is essential for transducing signals to epithelial cells. *Proc Natl Acad Sci U S A.* **1995**;92(17):7991-5.

71. **Navarro-Garcia F., Sonnested M., Teter K.** Host-Toxin Interactions Involving EspC and Pet, Two Serine Protease Autotransporters of the Enterobacteriaceae. *Toxins*. **2010**;2(5).
72. **Navarro-Garcia F., Canizalez-Roman A., Sui B.Q., Nataro J.P., Azamar Y.** The serine protease motif of EspC from enteropathogenic *Escherichia coli* produces epithelial damage by a mechanism different from that of Pet toxin from enteroaggregative *E. coli*. *Infect Immun*. **2004**;72(6):3609-21.
73. **Guignot J., Segura A., Tran Van Nhieu G.** The Serine Protease EspC from Enteropathogenic *Escherichia coli* Regulates Pore Formation and Cytotoxicity Mediated by the Type III Secretion System. *PLoS Pathog*. **2015**;11(7):e1005013.
74. **Abreu A.G., Bueris V., Porangaba T.M., Sircili M.P., Navarro-Garcia F., Elias W.P.** Autotransporter Protein-Encoding Genes of Diarrheagenic *Escherichia coli* Are Found in both Typical and Atypical Enteropathogenic *E. coli* Strains. *Applied and Environmental Microbiology*. **2013**;79(1):411-4.
75. **Desvaux M., Cooper L.M., Filenko N.A., Scott-Tucker A., Turner S.M., Cole J.A., et al.** The unusual extended signal peptide region of the type V secretion system is phylogenetically restricted. *FEMS Microbiol Lett*. **2006**;264(1):22-30.
76. **Henderson I.R., Navarro-Garcia F., Nataro J.P.** The great escape: structure and function of the autotransporter proteins. *Trends Microbiol*. **1998**;6(9):370-8.
77. **Leyton D.L., de Luna M., Sevastsyanovich Y.R., Tveen Jensen K., Browning D.F., Scott-Tucker A., et al.** The unusual extended signal peptide region is not required for secretion and function

of an *Escherichia coli* autotransporter. *FEMS Microbiol Lett.* **2010**;311(2):133-9.

78. **Pina-Pedrero S., Olvera A., Bensaid A.** The extended leader peptide of *Haemophilus parasuis* trimeric autotransporters conditions their protein expression in *Escherichia coli*. *Protein Expr Purif.* **2017**;133:15-24.

79. **Ariosa A., Lee J.H., Wang S., Saraogi I., Shan S.O.** Regulation by a chaperone improves substrate selectivity during cotranslational protein targeting. *Proc Natl Acad Sci U S A.* **2015**;112(25):E3169-78.

80. **Jong W.S., Luirink J.** The conserved extension of the Hbp autotransporter signal peptide does not determine targeting pathway specificity. *Biochem Biophys Res Commun.* **2008**;368(3):522-7.

81. **Junker M., Schuster C.C., McDonnell A.V., Sorg K.A., Finn M.C., Berger B., et al.** Pertactin beta-helix folding mechanism suggests common themes for the secretion and folding of autotransporter proteins. *Proc Natl Acad Sci U S A.* **2006**;103(13):4918-23.

82. **Yen Y.T., Kostakioti M., Henderson I.R., Stathopoulos C.** Common themes and variations in serine protease autotransporters. *Trends Microbiol.* **2008**;16(8):370-9.

83. **Dutta P.R., Cappello R., Navarro-Garcia F., Nataro J.P.** Functional comparison of serine protease autotransporters of Enterobacteriaceae. *Infect Immun.* **2002**;70(12):7105-13.

84. **Henderson I.R., Nataro J.P.** Virulence functions of autotransporter proteins. *Infect Immun.* **2001**;69(3):1231-43.

85. **Renn J.P., Clark P.L.** A conserved stable core structure in the passenger domain beta-helix of autotransporter virulence proteins. *Biopolymers.* **2008**;89(5):420-7.

86. **Kuhnel K., Diezmann D.** Crystal structure of the autochaperone region from the *Shigella flexneri* autotransporter IcsA. *J Bacteriol.* **2011**;193(8):2042-5.
87. **Ivie S.E., McClain M.S., Algood H.M., Lacy D.B., Cover T.L.** Analysis of a beta-helical region in the p55 domain of *Helicobacter pylori* vacuolating toxin. *BMC Microbiol.* **2010**;10:60.
88. **Ohnishi Y., Nishiyama M., Horinouchi S., Beppu T.** Involvement of the COOH-terminal pro-sequence of *Serratia marcescens* serine protease in the folding of the mature enzyme. *J Biol Chem.* **1994**;269(52):32800-6.
89. **Teh M.Y., Tran E.N.H., Morona R.** Absence of O antigen suppresses *Shigella flexneri* IcsA autochaperone region mutations. *Microbiology (Reading).* **2012**;158(Pt 11):2835-50.
90. **Oliver D.C., Huang G., Nodel E., Pleasance S., Fernandez R.C.** A conserved region within the *Bordetella pertussis* autotransporter BrkA is necessary for folding of its passenger domain. *Mol Microbiol.* **2003**;47(5):1367-83.
91. **Celik N., Webb C.T., Leyton D.L., Holt K.E., Heinz E., Gorrell R., et al.** A Bioinformatic Strategy for the Detection, Classification and Analysis of Bacterial Autotransporters. *PLoS One.* **2012**;8(7).
92. **Rojas-Lopez M., Zorgani M.A., Kelley L.A., Bailly X., Kajava A.V., Henderson I.R., et al.** Identification of the Autochaperone Domain in the Type Va Secretion System (T5aSS): Prevalent Feature of Autotransporters with a beta-Helical Passenger. *Front Microbiol.* **2017**;8:2607.
93. **Soprova Z., Sauri A., van Ulsen P., Tame J.R., den Blaauwen T., Jong W.S., et al.** A conserved aromatic residue in the

autochaperone domain of the autotransporter Hbp is critical for initiation of outer membrane translocation. *J Biol Chem.* **2010**;285(49):38224-33.

94. **Velarde J.J., Nataro J.P.** Hydrophobic residues of the autotransporter EspP linker domain are important for outer membrane translocation of its passenger. *J Biol Chem.* **2004**;279(30):31495-504.

95. **Dutta P.R., Sui B.Q., Nataro J.P.** Structure-function analysis of the enteroaggregative *Escherichia coli* plasmid-encoded toxin autotransporter using scanning linker mutagenesis. *J Biol Chem.* **2003**;278(41):39912-20.

96. **Junker M., Clark P.L.** Slow formation of aggregation-resistant beta-sheet folding intermediates. *Proteins.* **2010**;78(4):812-24.

97. **Renn J.P., Junker M., Besingi R.N., Braselmann E., Clark P.L.** ATP-independent control of autotransporter virulence protein transport via the folding properties of the secreted protein. *Chem Biol.* **2012**;19(2):287-96.

98. **Besingi R.N., Chaney J.L., Clark P.L.** An alternative outer membrane secretion mechanism for an autotransporter protein lacking a C-terminal stable core. *Mol Microbiol.* **2013**;90(5):1028-45.

99. **Kostakioti M., Stathopoulos C.** Role of the alpha-helical linker of the C-terminal translocator in the biogenesis of the serine protease subfamily of autotransporters. *Infect Immun.* **2006**;74(9):4961-9.

100. **Oomen C.J., van Ulsen P., van Gelder P., Feijen M., Tommassen J., Gros P.** Structure of the translocator domain of a bacterial autotransporter. *EMBO J.* **2004**;23(6):1257-66.

101. **Barnard T.J., Dautin N., Lukacik P., Bernstein H.D., Buchanan S.K.** Autotransporter structure reveals intra-barrel cleavage

followed by conformational changes. *Nat Struct Mol Biol.* **2007**;14(12):1214-20.

102. **van den Berg B.** Crystal structure of a full-length autotransporter. *J Mol Biol.* **2010**;396(3):627-33.

103. **Tajima N., Kawai F., Park S.Y., Tame J.R.** A novel intein-like autoproteolytic mechanism in autotransporter proteins. *J Mol Biol.* **2010**;402(4):645-56.

104. **Dautin N., Barnard T.J., Anderson D.E., Bernstein H.D.** Cleavage of a bacterial autotransporter by an evolutionarily convergent autocatalytic mechanism. *EMBO J.* **2007**;26(7):1942-52.

105. **Uversky V.N., Gillespie J.R., Fink A.L.** Why are "natively unfolded" proteins unstructured under physiologic conditions? *Proteins.* **2000**;41(3):415-27.

106. **Walsh I., Martin A.J., Di Domenico T., Tosatto S.C.** ESpritz: accurate and fast prediction of protein disorder. *Bioinformatics.* **2012**;28(4):503-9.

107. **Roussel-Jazede V., Van Gelder P., Sijbrandi R., Rutten L., Otto B.R., Luirink J., et al.** Channel properties of the translocator domain of the autotransporter Hbp of *Escherichia coli*. *Mol Membr Biol.* **2011**;28(3):158-70.

108. **Gawarzewski I., Smits S.H., Schmitt L., Jose J.** Structural comparison of the transport units of type V secretion systems. *Biol Chem.* **2013**;394(11):1385-98.

109. **Janakiraman A., Fixen K.R., Gray A.N., Niki H., Goldberg M.B.** A genome-scale proteomic screen identifies a role for DnaK in chaperoning of polar autotransporters in *Shigella*. *J Bacteriol.* **2009**;191(20):6300-11.

110. **Sachelaru I., Petriman N.A., Kudva R., Koch H.G.** Dynamic Interaction of the Sec Translocon with the Chaperone PpiD. The *Journal of Biological Chemistry*. **2014**;289(31):21706-15.
111. **Komarudin A.G., Driessen A.J.M.** SecA-Mediated Protein Translocation through the SecYEG Channel. *Microbiology Spectrum*. **2019**.
112. **Szabady R.L., Peterson J.H., Skillman K.M., Bernstein H.D.** An unusual signal peptide facilitates late steps in the biogenesis of a bacterial autotransporter. *Proc Natl Acad Sci U S A*. **2005**;102(1):221-6.
113. **Desvaux M., Scott-Tucker A., Turner S.M., Cooper L.M., Huber D., Nataro J.P., et al.** A conserved extended signal peptide region directs posttranslational protein translocation via a novel mechanism. *Microbiology (Reading)*. **2007**;153(Pt 1):59-70.
114. **Jong W.S., ten Hagen-Jongman C.M., Ruijter E., Orru R.V., Genevaux P., Luirink J.** YidC is involved in the biogenesis of the secreted autotransporter hemoglobin protease. *J Biol Chem*. **2010**;285(51):39682-90.
115. **Brandon L.D., Goehring N., Janakiraman A., Yan A.W., Wu T., Beckwith J., et al.** IcsA, a polarly localized autotransporter with an atypical signal peptide, uses the Sec apparatus for secretion, although the Sec apparatus is circumferentially distributed. *Mol Microbiol*. **2003**;50(1):45-60.
116. **Jauss B., Petriman N.A., Drepper F., Franz L., Sachelaru I., Welte T., et al.** Noncompetitive binding of PpiD and YidC to the SecYEG translocon expands the global view on the SecYEG interactome in *Escherichia coli*. *J Biol Chem*. **2019**;294(50):19167-83.
117. **Ieva R., Tian P., Peterson J.H., Bernstein H.D.** Sequential and spatially restricted interactions of assembly factors with an

autotransporter β domain. *Proceedings of the National Academy of Sciences*. **2011**;108(31):E383-E91.

118. **Rizzitello A.E., Harper J.R., Silhavy T.J.** Genetic evidence for parallel pathways of chaperone activity in the periplasm of *Escherichia coli*. *J Bacteriol*. **2001**;183(23):6794-800.

119. **Sklar J.G., Wu T., Kahne D., Silhavy T.J.** Defining the roles of the periplasmic chaperones SurA, Skp, and DegP in *Escherichia coli*. *Genes Dev*. **2007**;21(19):2473-84.

120. **Purdy G.E., Fisher C.R., Payne S.M.** IcsA Surface Presentation in *Shigella flexneri* Requires the Periplasmic Chaperones DegP, Skp, and SurA ∇ . *Journal of Bacteriology*. **2007**;189(15):5566-73.

121. **Ruiz-Perez F., Henderson I.R., Leyton D.L., Rossiter A.E., Zhang Y., Nataro J.P.** Roles of Periplasmic Chaperone Proteins in the Biogenesis of Serine Protease Autotransporters of Enterobacteriaceae. *Journal of Bacteriology*. **2009**;191(21):6571-83.

122. **Sauri A., Soprova Z., Wickstrom D., de Gier J.W., Van der Schors R.C., Smit A.B., et al.** The Bam (Omp85) complex is involved in secretion of the autotransporter haemoglobin protease. *Microbiology*. **2009**;155(12).

123. **Tapia-Pastrana G., Chavez-Duenas L., Lanz-Mendoza H., Teter K., Navarro-Garcia F.** VirK is a periplasmic protein required for efficient secretion of plasmid-encoded toxin from enteroaggregative *Escherichia coli*. *Infect Immun*. **2012**;80(7):2276-85.

124. **Qu J., Mayer C., Behrens S., Holst O., Kleinschmidt J.H.** The trimeric periplasmic chaperone Skp of *Escherichia coli* forms 1:1 complexes with outer membrane proteins via hydrophobic and electrostatic interactions. *J Mol Biol*. **2007**;374(1):91-105.

125. **Jong W.S., ten Hagen-Jongman C.M., den Blaauwen T., Slotboom D.J., Tame J.R., Wickstrom D., et al.** Limited tolerance towards folded elements during secretion of the autotransporter Hbp. *Mol Microbiol.* **2007**;63(5):1524-36.
126. **Spiess C., Beil A., Ehrmann M.** A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. *Cell.* **1999**;97(3):339-47.
127. **Brasemann E., Chaney J.L., Champion M.M., Clark P.L.** DegP Chaperone Suppresses Toxic Inner Membrane Translocation Intermediates. *PLoS One.* **2016**;11(9):e0162922.
128. **Ruiz-Perez F., Henderson I.R., Nataro J.P.** Interaction of FkpA, a peptidyl-prolyl cis/trans isomerase with EspP autotransporter protein. *Gut Microbes.* **2010**;1(5):339-44.
129. **Yan Z., Hussain S., Wang X., Bernstein H.D., Bardwell J.C.A.** Chaperone OsmY facilitates the biogenesis of a major family of autotransporters. *Mol Microbiol.* **2019**;112(5):1373-87.
130. **Nakata N., Sasakawa C., Okada N., Tobe T., Fukuda I., Suzuki T., et al.** Identification and characterization of *virK*, a virulence-associated large plasmid gene essential for intercellular spreading of *Shigella flexneri*. *Mol Microbiol.* **1992**;6(16):2387-95.
131. **Wagner J.K., Heindl J.E., Gray A.N., Jain S., Goldberg M.B.** Contribution of the periplasmic chaperone Skp to efficient presentation of the autotransporter IcsA on the surface of *Shigella flexneri*. *J Bacteriol.* **2009**;191(3):815-21.
132. **Purdy G.E., Hong M., Payne S.M.** *Shigella flexneri* DegP facilitates IcsA surface expression and is required for efficient intercellular spread. *Infect Immun.* **2002**;70(11):6355-64.

133. **Ruiz-Perez F., Henderson I.R., Nataro J.P.** Interaction of FkpA, a peptidyl-prolyl cis/trans isomerase with EspP autotransporter protein. *Gut Microbes*. **2010**;1(5):339-44.
134. **Ulrich T., Oberhettinger P., Schutz M., Holzer K., Ramms A.S., Linke D., et al.** Evolutionary conservation in biogenesis of beta-barrel proteins allows mitochondria to assemble a functional bacterial trimeric autotransporter protein. *J Biol Chem*. **2014**;289(43):29457-70.
135. **Muller J.E., Papic D., Ulrich T., Grin I., Schutz M., Oberhettinger P., et al.** Mitochondria can recognize and assemble fragments of a beta-barrel structure. *Mol Biol Cell*. **2011**;22(10):1638-47.
136. **Veiga E., de Lorenzo V., Fernandez L.A.** Structural tolerance of bacterial autotransporters for folded passenger protein domains. *Mol Microbiol*. **2004**;52(4):1069-80.
137. **Bodelon G., Marin E., Fernandez L.A.** Role of periplasmic chaperones and BamA (YaeT/Omp85) in folding and secretion of intimin from enteropathogenic *Escherichia coli* strains. *J Bacteriol*. **2009**;191(16):5169-79.
138. **Mogensen J.E., Kleinschmidt J.H., Schmidt M.A., Otzen D.E.** Misfolding of a bacterial autotransporter. *Protein Sci*. **2005**;14(11):2814-27.
139. **Ercoli G., Tani C., Pezzicoli A., Vacca I., Martinelli M., Pecetta S., et al.** LytM proteins play a crucial role in cell separation, outer membrane composition, and pathogenesis in nontypeable *Haemophilus influenzae*. *mBio*. **2015**;6(2):e02575.
140. **Xue Z., Pang Y., Quan S.** Revisiting the functions of periplasmic chaperones in the quality control of the autotransporter Ag43 using a

phenotypically homogeneous *Escherichia coli* strain. *Biochem Biophys Res Commun.* **2022**;591:37-43.

141. **Jain S., Goldberg M.B.** Requirement for YaeT in the outer membrane assembly of autotransporter proteins. *J Bacteriol.* **2007**;189(14):5393-8.

142. **Han L., Zheng J., Wang Y., Yang X., Liu Y., Sun C., et al.** Structure of the BAM complex and its implications for biogenesis of outer-membrane proteins. *Nat Struct Mol Biol.* **2016**;23(3):192-6.

143. **Albrecht R., Schutz M., Oberhettinger P., Faulstich M., Bermejo I., Rudel T., et al.** Structure of BamA, an essential factor in outer membrane protein biogenesis. *Acta Crystallogr D Biol Crystallogr.* **2014**;70(Pt 6):1779-89.

144. **Bakelar J., Buchanan S.K., Noinaj N.** The structure of the beta-barrel assembly machinery complex. *Science.* **2016**;351(6269):180-6.

145. **Voulhoux R., Bos M.P., Geurtsen J., Mols M., Tommassen J.** Role of a highly conserved bacterial protein in outer membrane protein assembly. *Science.* **2003**;299(5604):262-5.

146. **Rossiter A.E., Leyton D.L., Tveen-Jensen K., Browning D.F., Sevastsyanovich Y., Knowles T.J., et al.** The essential β -barrel assembly machinery complex components BamD and BamA are required for autotransporter biogenesis. *J Bacteriol.* **2011**;193(16):4250-3.

147. **Rooke J.L., Icke C., Wells T.J., Rossiter A.E., Browning D.F., Morris F.C., et al.** BamA and BamD Are Essential for the Secretion of Trimeric Autotransporter Adhesins. *Front Microbiol.* **2021**;12:628879.

148. **Lehr U., Schutz M., Oberhettinger P., Ruiz-Perez F., Donald J.W., Palmer T., et al.** C-terminal amino acid residues of the trimeric

autotransporter adhesin YadA of *Yersinia enterocolitica* are decisive for its recognition and assembly by BamA. *Mol Microbiol.* **2010**;78(4):932-46.

149. **Selkrig J., Mosbahi K., Webb C.T., Belousoff M.J., Perry A.J., Wells T.J., et al.** Discovery of an archetypal protein transport system in bacterial outer membranes. *Nature Structural & Molecular Biology.* **2012**;19(5):506.

150. **Kang'ethe W., Bernstein H.D.** Stepwise folding of an autotransporter passenger domain is not essential for its secretion. *J Biol Chem.* **2013**;288(49):35028-38.

151. **Ieva R., Skillman K.M., Bernstein H.D.** Incorporation of a polypeptide segment into the beta-domain pore during the assembly of a bacterial autotransporter. *Mol Microbiol.* **2008**;67(1):188-201.

152. **Sikdar R., Peterson J.H., Anderson D.E., Bernstein H.D.** Folding of a bacterial integral outer membrane protein is initiated in the periplasm. *Nat Commun.* **2017**;8(1):1309.

153. **Robert V., Volokhina E.B., Senf F., Bos M.P., Van Gelder P., Tommassen J.** Assembly factor Omp85 recognizes its outer membrane protein substrates by a species-specific C-terminal motif. *PLoS Biol.* **2006**;4(11):e377.

154. **Schiffrin B., Machin J.M., Karamanos T.K., Zhuravleva A., Brockwell D.J., Radford S.E., et al.** Dynamic interplay between the periplasmic chaperone SurA and the BAM complex in outer membrane protein folding. *Commun Biol.* **2022**;5(1):560.

155. **Pavlova O., Peterson J.H., Ieva R., Bernstein H.D.** Mechanistic link between β barrel assembly and the initiation of autotransporter secretion. *Proceedings of the National Academy of Sciences.* **2013**;110(10):E938-E47.

156. **Tomasek D., Rawson S., Lee J., Wzorek J.S., Harrison S.C., Li Z., et al.** Structure of a nascent membrane protein as it folds on the BAM complex. *Nature*. **2020**;583(7816):473-8.
157. **Lundquist K., Billings E., Bi M., Wellnitz J., Noinaj N.** The assembly of beta-barrel membrane proteins by BAM and SAM. *Mol Microbiol*. **2021**;115(3):425-35.
158. **Yuan X., Johnson M.D., Zhang J., Lo A.W., Schembri M.A., Wijeyewickrema L.C., et al.** Molecular basis for the folding of beta-helical autotransporter passenger domains. *Nat Commun*. **2018**;9(1):1395.
159. **Peterson J.H., Hussain S., Bernstein H.D.** Identification of a novel post-insertion step in the assembly of a bacterial outer membrane protein. *Mol Microbiol*. **2018**;110(1):143-59.
160. **Phan T.H., Kuijl C., Huynh D.T., Jong W.S.P., Luirink J., van Ulsen P.** Overproducing the BAM complex improves secretion of difficult-to-secrete recombinant autotransporter chimeras. *Microb Cell Fact*. **2021**;20(1):176.
161. **Leo J.C., Linke D.** A unified model for BAM function that takes into account type Vc secretion and species differences in BAM composition. *AIMS Microbiol*. **2018**;4(3):455-68.
162. **Albenne C., Ieva R.** Job contenders: roles of the beta-barrel assembly machinery and the translocation and assembly module in autotransporter secretion. *Mol Microbiol*. **2017**;106(4):505-17.
163. **Heinz E., Stubenrauch C.J., Grinter R., Croft N.P., Purcell A.W., Strugnell R.A., et al.** Conserved Features in the Structure, Mechanism, and Biogenesis of the Inverse Autotransporter Protein Family. *Genome Biol Evol*. **2016**;8(6):1690-705.

164. **Chu Y., Wang Z., Weigold S., Norrell D., Fan E.** TtOmp85, a single Omp85 member protein functions as a beta-barrel protein insertase and an autotransporter translocase without any accessory proteins. *Biochem Biophys Res Commun.* **2021**;552:73-7.
165. **Selkrig J., Mosbahi K., Webb C.T., Belousoff M.J., Perry A.J., Wells T.J., et al.** Discovery of an archetypal protein transport system in bacterial outer membranes. *Nat Struct Mol Biol.* **2012**;19(5):506-10, S1.
166. **Shen H.H., Leyton D.L., Shiota T., Belousoff M.J., Noinaj N., Lu J., et al.** Reconstitution of a nanomachine driving the assembly of proteins into bacterial outer membranes. *Nat Commun.* **2014**;5:5078.
167. **Norell D., Heuck A., Tran-Thi T.A., Gotzke H., Jacob-Dubuisson F., Clausen T., et al.** Versatile in vitro system to study translocation and functional integration of bacterial outer membrane proteins. *Nat Commun.* **2014**;5:5396.
168. **Stubenrauch C., Grinter R., Lithgow T.** The modular nature of the beta-barrel assembly machinery, illustrated in *Borrelia burgdorferi*. *Mol Microbiol.* **2016**;102(5):753-6.
169. **Ramos-Leon F., Ramamurthi K.S.** Cytoskeletal proteins: lessons learned from bacteria. *Phys Biol.* **2022**;19(2).
170. **Jones L.J., Carballido-Lopez R., Errington J.** Control of cell shape in bacteria: helical, actin-like filaments in *Bacillus subtilis*. *Cell.* **2001**;104(6):913-22.
171. **Hussain S., Wivagg C.N., Szwedziak P., Wong F., Schaefer K., Izore T., et al.** MreB filaments align along greatest principal membrane curvature to orient cell wall synthesis. *Elife.* **2018**;7.
172. **van Teeffelen S., Wang S., Furchtgott L., Huang K.C., Wingreen N.S., Shaevitz J.W., et al.** The bacterial actin MreB rotates,

and rotation depends on cell-wall assembly. *PNAS*.

2011;108(38):15822–7.

173. **Garner E.C., Bernard R., Wang W., Zhuang X., Rudner D.Z., Mitchison T.** Coupled, circumferential motions of the cell wall synthesis machinery and MreB filaments in *B. subtilis*. *Science*. **2011**;333(6039):222-5.

174. **Ursell T.S., Nguyen J., Monds R.D., Colavin A., Billings G., Ouzounov N., et al.** Rod-like bacterial shape is maintained by feedback between cell curvature and cytoskeletal localization. *Proc Natl Acad Sci U S A*. **2014**;111(11):E1025-34.

175. **Li H., Gao T.** MreB and MreC act as the geometric moderators of the cell wall synthetic machinery in *Thermus thermophiles*. *Microbiol Res*. **2021**;243:126655.

176. **Cowles K.N., Gitai Z.** Surface association and the MreB cytoskeleton regulate pilus production, localization and function in *Pseudomonas aeruginosa*. *Mol Microbiol*. **2010**;76(6):1411-26.

177. **Strahl H., Burmann F., Hamoen L.W.** The actin homologue MreB organizes the bacterial cell membrane. *Nat Commun*. **2014**;5:3442.

178. **Oswald F., Varadarajan A., Lill H., Peterman E.J., Bollen Y.J.** MreB-Dependent Organization of the *E. coli* Cytoplasmic Membrane Controls Membrane Protein Diffusion. *Biophys J*. **2016**;110(5):1139-49.

179. **Kurita K., Kato F., Shiomi D.** Alteration of Membrane Fluidity or Phospholipid Composition Perturbs Rotation of MreB Complexes in *Escherichia coli*. *Front Mol Biosci*. **2020**;7:582660.

180. **Laloux G., Jacobs-Wagner C.** How do bacteria localize proteins to the cell pole? *J Cell Sci*. **2014**;127(Pt 1):11-9.

181. **Bowman G.R., Lyuksyutova A.I., Shapiro L.** Bacterial polarity. *Curr Opin Cell Biol.* **2011**;23(1):71-7.
182. **Scott M.E., Dossani Z.Y., Sandkvist M.** Directed polar secretion of protease from single cells of *Vibrio cholerae* via the type II secretion pathway. *Proc Natl Acad Sci U S A.* **2001**;98(24):13978-83.
183. **Chakravortty D., Rohde M., Jäger L., Deiwick J., Hensel M.** Formation of a novel surface structure encoded by Salmonella Pathogenicity Island 2. *EMBO J.* **2005**;24(11):2043-52.
184. **Morgan J.K., Luedtke B.E., Shaw E.I.** Polar localization of the *Coxiella burnetii* type IVB secretion system. *FEMS Microbiol Lett.* **2010**;305(2):177-83.
185. **Charles M., Perez M., Kobil J.H., Goldberg M.B.** Polar targeting of *Shigella* virulence factor IcsA in Enterobacteriaceae and *Vibrio*. *Proc Natl Acad Sci U S A.* **2001**;98(17):9871-6.
186. **Aguilar J., Zupan J., Cameron T.A., Zambryski P.C.** *Agrobacterium* type IV secretion system and its substrates form helical arrays around the circumference of virulence-induced cells. *Proc Natl Acad Sci U S A.* **2010**;107(8):3758-63.
187. **Jain S., van Ulsen P., Benz I., Schmidt M.A., Fernandez R., Tommassen J., et al.** Polar Localization of the Autotransporter Family of Large Bacterial Virulence Proteins. *Journal of Bacteriology.* **2006**;188(13):4841-50.
188. **Fixen K.R., Janakiraman A., Garrity S., Slade D.J., Gray A.N., Karahan N., et al.** Genetic reporter system for positioning of proteins at the bacterial pole. *mBio.* **2012**;3(2).

189. **Ebersbach G., Jacobs-Wagner C.** Exploration into the spatial and temporal mechanisms of bacterial polarity. *Trends Microbiol.* **2007**;15(3):101-8.
190. **Shih Y.L., Kawagishi I., Rothfield L.** The MreB and Min cytoskeletal-like systems play independent roles in prokaryotic polar differentiation. *Mol Microbiol.* **2005**;58(4):917-28.
191. **Nilsen T., Yan A.W., Gale G., Goldberg M.B.** Presence of multiple sites containing polar material in spherical *Escherichia coli* cells that lack MreB. *J Bacteriol.* **2005**;187(17):6187-96.
192. **Cambre A., Aertsen A.** Bacterial Vivisection: How Fluorescence-Based Imaging Techniques Shed a Light on the Inner Workings of Bacteria. *Microbiol Mol Biol Rev.* **2020**;84(4).
193. **Zakeri B., Fierer J.O., Celik E., Chittock E.C., Schwarz-Linek U., Moy V.T., et al.** Peptide tag forming a rapid covalent bond to a protein, through engineering a bacterial adhesin. *Proc Natl Acad Sci U S A.* **2012**;109(12):E690-7.
194. **Hatlem D., Trunk T., Linke D., Leo J.C.** Catching a SPY: Using the SpyCatcher-SpyTag and Related Systems for Labeling and Localizing Bacterial Proteins. *International Journal of Molecular Sciences.* **2019**;20(9).
195. **Chauhan N., Hatlem D., Orwick-Rydmark M., Schneider K., Floetenmeyer M., van Rossum B., et al.** Insights into the autotransport process of a trimeric autotransporter, Yersinia Adhesin A (YadA). *Mol Microbiol.* **2019**;111(3):844-62.
196. **Narancic T., Almahboub S.A., O'Connor K.E.** Unnatural amino acids: production and biotechnological potential. *World J Microbiol Biotechnol.* **2019**;35(4):67.

197. **Young T.S., Ahmad I., Yin J.A., Schultz P.G.** An enhanced system for unnatural amino acid mutagenesis in *E. coli*. *J Mol Biol.* **2010**;395(2):361-74.
198. **Shoham M., Greenberg M.** Preventing the spread of infectious diseases: antivirulents versus antibiotics. *Future Microbiology.* **2017**;12(5):365-8.
199. **Prezioso S.M., Brown N.E., Goldberg J.B.** Elfamycins: inhibitors of elongation factor-Tu. *Mol Microbiol.* **2017**;106(1):22-34.
200. **Maehr H., Smallheer J., Schaffner C.P.** Chemistry of the gentamicins. III. A comment of the structure of gentamicin A. *J Antibiot (Tokyo).* **1980**;33(11):1380-2.
201. **McHugh R.E., Munnoch J.T., Braes R.E., McKean I.J.W., Giard J., Taladriz-Sender A., et al.** Biosynthesis of Aurodox, a Type III Secretion System Inhibitor from *Streptomyces goldiniensis*. *Appl Environ Microbiol.* **2022**;88(15):e0069222.
202. **Kavaliauskas D., Nissen P., Knudsen C.R.** The busiest of all ribosomal assistants: elongation factor Tu. *Biochemistry.* **2012**;51(13):2642-51.
203. **Vogele L., Palm G.J., Mesters J.R., Hilgenfeld R.** Conformational change of elongation factor Tu (EF-Tu) induced by antibiotic binding. Crystal structure of the complex between EF-Tu, GDP and aurodox. *J Biol Chem.* **2001**;276(20):17149-55.
204. **Kimura K., Iwatsuki M., Nagai T., Matsumoto A., Takahashi Y., Shiomi K., et al.** A small-molecule inhibitor of the bacterial type III secretion system protects against *in vivo* infection with *Citrobacter rodentium*. *J Antibiot (Tokyo).* **2011**;64(2):197-203.

205. **McHugh R.E., O'Boyle N., Connolly J.P.R., Hoskisson P.A., Roe A.J.** Characterization of the Mode of Action of Aurodox, a Type III Secretion System Inhibitor from *Streptomyces goldiniensis*. *Infect Immun.* **2019**;87(2).
206. **Ashawesh M.M., Markus R., Penfold C.N., Hardie K.R.** Autotransporter secretion exploits the bacterial actin-homologue. **2018**. [Available at: <https://www.biorxiv.org/content/10.1101/305441v2.full>].
207. **Ashawesh M.** Investigation of the spiral secretion pattern of the serine protease autotransporter, EspC using innovative fluorescent labelling approaches: University of Nottingham; **2016**.
208. **Popp D., Narita A., Maeda K., Fujisawa T., Ghoshdastider U., Iwasa M., et al.** Filament Structure, Organization, and Dynamics in MreB Sheets. *The Journal of Biological Chemistry.* **2010**;285(21):15858–65.
209. **Matsumoto K., Hara H., Fishov I., Mileykovskaya E., Norris V.** The membrane: transertion as an organizing principle in membrane heterogeneity. *Frontiers in Microbiology.* **2015**;6.
210. **Shiomi D., Yoshimoto M., Homma M., Kawagishi H.** Helical distribution of the bacterial chemoreceptor via colocalization with the Sec protein translocation machinery. *Molecular Microbiology.* **2006**;60(4):894-906.
211. **Govindarajan S., Amster-Choder O.** The bacterial Sec system is required for the organization and function of the MreB cytoskeleton. *PLoS Genetics.* **2017**;13(9).
212. **Jensen K.F.** The *Escherichia coli* K-12 "wild types" W3110 and MG1655 have an *rph* frameshift mutation that leads to pyrimidine starvation due to low pyrE expression levels. *J Bacteriol.* **1993**;175(11):3401-7.

213. **Oliver D.B., Beckwith J.** Identification of a new gene (*secA*) and gene product involved in the secretion of envelope proteins in *Escherichia coli*. *J Bacteriol.* **1982**;150(2):686-91.
214. **Shiba K., Ito K., Yura T.** Suppressors of the *secY24* mutation: identification and characterization of additional *ssy* genes in *Escherichia coli*. *J Bacteriol.* **1986**;166(3):849-56.
215. **Gardel C., Benson S., Hunt J., Michaelis S., Beckwith J.** *secD*, a new gene involved in protein export in *Escherichia coli*. *J Bacteriol.* **1987**;169(3):1286-90.
216. **Iguchi A., Thomson N.R., Ogura Y., Saunders D., Ooka T., Henderson I.R., et al.** Complete genome sequence and comparative genome analysis of enteropathogenic *Escherichia coli* O127:H6 strain E2348/69. *J Bacteriol.* **2009**;191(1):347-54.
217. **Durfee T., Nelson R., Baldwin S., Plunkett G. 3rd, Burland V., Mau B., et al.** The complete genome sequence of *Escherichia coli* DH10B: insights into the biology of a laboratory workhorse. *J Bacteriol.* **2008**;190(7):2597-606.
218. **Arnold L.** Characterisation of factors involved in the secretion of EspC in enteropathogenic *Escherichia coli* : University of Nottingham; **2003**.
219. **Sanchez-Villamil J.I., Navarro-Garcia F., Castillo-Romero A., Gutierrez-Gutierrez F., Tapia D., Tapia-Pastrana G.** Curcumin Blocks Cytotoxicity of Enteropathogenic and Enteropathogenic *Escherichia coli* by Blocking Pet and EspC Proteolytic Release From Bacterial Outer Membrane. *Front Cell Infect Microbiol.* **2019**;9:334.
220. **Leverton L.Q., Kaper J.B.** Temporal expression of enteropathogenic *Escherichia coli* virulence genes in an *in vitro* model of infection. *Infect Immun.* **2005**;73(2):1034-43.

221. **Bishop K.** Dissecting protein-protein interactions facilitating secretion of the virulence factor EspC by enteropathogenic *Escherichia coli*. **2009**.
222. **Farrell I.S., Toroney R., Hazen J.L., Mehl R.A., Chin J.W.** Photo-cross-linking interacting proteins with a genetically encoded benzophenone. *Nature Methods*. **2005**;2(5):377.
223. **Bolte S., Cordelieres F.P.** A guided tour into subcellular colocalization analysis in light microscopy. *J Microsc*. **2006**;224(Pt 3):213-32.
224. **Shaner N.C., Campbell R.E., Steinbach P.A., Giepmans B.N.G., Palmer A.E., Tsien R.Y.** Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma sp.* red fluorescent protein. *Nature Biotechnology*. **2004**;22(12):1567-72.
225. **Pena M.M., Teper D., Ferreira H., Wang N., Sato K.U., Ferro M.I.T., et al.** mCherry fusions enable the subcellular localization of periplasmic and cytoplasmic proteins in *Xanthomonas sp.* PLoS One. **2020**;15(7):e0236185.
226. **Skoog K., Soderstrom B., Widengren J., von Heijne G., Daley D.O.** Sequential closure of the cytoplasm and then the periplasm during cell division in *Escherichia coli*. *J Bacteriol*. **2012**;194(3):584-6.
227. **Cranfill P.J., Sell B.R., Baird M.A., Allen J.R., Lavagnino Z., de Gruiter H.M., et al.** Quantitative assessment of fluorescent proteins. *Nat Methods*. **2016**;13(7):557-62.
228. **Landgraf D., Okumus B., Chien P., Baker T.A., Paulsson J.** Segregation of molecules at cell division reveals native protein localization. *Nat Methods*. **2012**;9(5):480-2.

229. **Adams S.R., Campbell R.E., Gross L.A., Martin B.R., Walkup G.K., Yao Y., et al.** New biarsenical ligands and tetracysteine motifs for protein labeling *in vitro* and *in vivo*: synthesis and biological applications. *J Am Chem Soc.* **2002**;124(21):6063-76.
230. **Bohl C., Pomorski A., Seemann S., Knospe A.M., Zheng C., Krezel A., et al.** Fluorescent probes for selective protein labeling in lysosomes: a case of alpha-galactosidase A. *FASEB J.* **2017**;31(12):5258-67.
231. **Hoffmann C., Gaietta G., Zürn A., Adams S.R., Terrillon S., Ellisman M.H., et al.** Fluorescent labeling of tetracysteine-tagged proteins in intact cells. *Nat Protoc.* **2010**;5(10):1666-77.
232. **Wrobel A., Saragliadis A., Perez-Ortega J., Sittman C., Gottig S., Liskiewicz K., et al.** The inverse autotransporters of *Yersinia ruckeri*, Yrlnv and Yrllm, contribute to biofilm formation and virulence. *Environ Microbiol.* **2020**;22(7):2939-55.
233. **Maurer J., Jose J., Meyer T.F.** Autodisplay: one-component system for efficient surface display and release of soluble recombinant proteins from *Escherichia coli*. *J Bacteriol.* **1997**;179(3):794-804.
234. **Ramesh B., Sendra V.G., Cirino P.C., Varadarajan N.** Single-cell characterization of autotransporter-mediated *Escherichia coli* surface display of disulfide bond-containing proteins. *J Biol Chem.* **2012**;287(46):38580-9.
235. **Gallus S., Mittmann E., Rabe K.S.** A Modular System for the Rapid Comparison of Different Membrane Anchors for Surface Display on *Escherichia coli*. *Chembiochem.* **2022**;23(2):e202100472.
236. **Khalil H.S., Ogaard J., Leo J.C.** Coaggregation properties of trimeric autotransporter adhesins. *Microbiologyopen.* **2020**;9(10):e1109.

237. **Valle J., Mabbett A.N., Ulett G.C., Toledo-Arana A., Wecker K., Totsika M., et al.** UpaG, a new member of the trimeric autotransporter family of adhesins in uropathogenic *Escherichia coli*. *J Bacteriol.* **2008**;190(12):4147-61.
238. **Vieira P.C.G., Espinoza-Culupu A.O., Nepomuceno R., Alves M.R., Lebrun I., Elias W.P., et al.** Secreted autotransporter toxin (Sat) induces cell damage during enteroaggregative *Escherichia coli* infection. *PLoS One.* **2020**;15(2):e0228959.
239. **Lu Q., Xu Y., Yao Q., Niu M., Shao F.** A polar-localized iron-binding protein determines the polar targeting of *Burkholderia* BimA autotransporter and actin tail formation. *Cell Microbiol.* **2015**;17(3):408-24.
240. **Kingsley R.A., Santos R.L., Kestra A.M., Adams L.G., Baumler A.J.** *Salmonella enterica* serotype Typhimurium ShdA is an outer membrane fibronectin-binding protein that is expressed in the intestine. *Mol Microbiol.* **2002**;43(4):895-905.
241. **Pokharel P., Diaz J.M., Bessaiah H., Houle S., Guerrero-Barrera A.L., Dozois C.M.** The Serine Protease Autotransporters TagB, TagC, and Sha from Extraintestinal Pathogenic *Escherichia coli* Are Internalized by Human Bladder Epithelial Cells and Cause Actin Cytoskeletal Disruption. *Int J Mol Sci.* **2020**;21(9).
242. **Diaz J.M., Dozois C.M., Avelar-Gonzalez F.J., Hernandez-Cuellar E., Pokharel P., de Santiago A.S., et al.** The Vacuolating Autotransporter Toxin (Vat) of *Escherichia coli* Causes Cell Cytoskeleton Changes and Produces Non-lysosomal Vacuole Formation in Bladder Epithelial Cells. *Front Cell Infect Microbiol.* **2020**;10:299.

243. **Vidal J.E., Navarro-Garcia F.** EspC translocation into epithelial cells by enteropathogenic *Escherichia coli* requires a concerted participation of type V and III secretion systems. *Cell Microbiol.* **2008**;10(10):1975-86.
244. **Varadi M., Anyango S., Deshpande M., Nair S., Natassia C., Yordanova G., et al.** AlphaFold Protein Structure Database: massively expanding the structural coverage of protein-sequence space with high-accuracy models. *Nucleic Acids Res.* **2022**;50(D1):D439-D44.
245. **Zhang Z., Schwartz S., Wagner L., Miller W.** A greedy algorithm for aligning DNA sequences. *J Comput Biol.* **2000**;7(1-2):203-14.
246. **Yang Z., Zhang L., Zhang Y., Zhang T., Feng Y., Lu X., et al.** Highly efficient production of soluble proteins from insoluble inclusion bodies by a two-step-denaturing and refolding method. *PLoS One.* **2011**;6(7):e22981.
247. **Bindels D.S., Haarbosch L., van Weeren L., Postma M., Wiese K.E., Mastop M., et al.** mScarlet: a bright monomeric red fluorescent protein for cellular imaging. *Nat Methods.* **2017**;14(1):53-6.
248. **Djakovic A., Hinde E., MacKichan C., Carballido-Lopez R.** Dynamic Organization of SecA and SecY Secretion Complexes in the *B. subtilis* Membrane. *PLoS One.* **2016**;11(6):e0157899.
249. **Campo N., Tjalsma H., Buist G., Stepniak D., Meijer M., Veenhuis M., et al.** Subcellular sites for bacterial protein export. *Mol Microbiol.* **2004**;53(6):1583-99.
250. **Kim S.Y., Gitai Z., Kinkhabwala A., Shapiro L., Moerner W.E.** Single molecules of the bacterial actin MreB undergo directed treadmilling motion in *Caulobacter crescentus*. *Proc Natl Acad Sci U S A.* **2006**;103(29):10929-34.

251. **McCausland J.W., Yang X., Squyres G.R., Lyu Z., Bruce K.E., Lamanna M.M., et al.** Treadmilling FtsZ polymers drive the directional movement of sPG-synthesis enzymes via a Brownian ratchet mechanism. *Nat Commun.* **2021**;12(1):609.
252. **Yang X., Lyu Z., Miguel A., McQuillen R., Huang K.C., Xiao J.** GTPase activity-coupled treadmilling of the bacterial tubulin FtsZ organizes septal cell wall synthesis. *Science.* **2017**;355(6326):744-7.
253. **van den Ent F., Izore T., Bharat T.A., Johnson C.M., Lowe J.** Bacterial actin MreB forms antiparallel double filaments. *Elife.* **2014**;3:e02634.
254. **Dominguez-Escobar J., Chastanet A., Crevenna A.H., Fromion V., Wedlich-Soldner R., Carballido-Lopez R.** Processive movement of MreB-associated cell wall biosynthetic complexes in bacteria. *Science.* **2011**;333(6039):225-8.
255. **Gitai Z., Dye N.A., Reisenauer A., Wachi M., Shapiro L.** MreB actin-mediated segregation of a specific region of a bacterial chromosome. *Cell.* **2005**;120(3):329-41.
256. **Vats P., Rothfield L.** Duplication and segregation of the actin (MreB) cytoskeleton during the prokaryotic cell cycle. *Proc Natl Acad Sci U S A.* **2007**;104(45):17795-800.
257. **Kruse T., Moller-Jensen J., Lobner-Olesen A., Gerdes K.** Dysfunctional MreB inhibits chromosome segregation in *Escherichia coli*. *EMBO J.* **2003**;22(19):5283-92.
258. **Fiebig A., Theriot J.A.** Bacteria make tracks to the pole. *Proc Natl Acad Sci U S A.* **2004**;101(23):8510-1.
259. **Gitai Z., Dye N., Shapiro L.** An actin-like gene can determine cell polarity in bacteria. *Proc Natl Acad Sci U S A.* **2004**;101(23):8643-8.

260. **Treuner-Lange A., Macia E., Guzzo M., Hot E., Faure L.M., Jakobczak B., et al.** The small G-protein MglA connects to the MreB actin cytoskeleton at bacterial focal adhesions. *J Cell Biol.* **2015**;210(2):243-56.
261. **Bowman G.R., Comolli L.R., Zhu J., Eckart M., Koenig M., Downing K.H., et al.** A polymeric protein anchors the chromosomal origin/ParB complex at a bacterial cell pole. *Cell.* **2008**;134(6):945-55.
262. **Gustafsson M.G.L., Allen J.R., Davidson M.W.** Super Resolution Structured Illumination Microscopy: Zeiss; [Available from: <https://zeiss-campus.magnet.fsu.edu/print/superresolution/supersim-print.html>].
263. **Hussein K.H., Park K.M., Lee Y.S., Woo J.S., Kang B.J., Choi K.Y., et al.** New insights into the pros and cons of cross-linking decellularized bioartificial organs. *Int J Artif Organs.* **2017**;40(4):136-41.
264. **Hoffman E.A., Frey B.L., Smith L.M., Auble D.T.** Formaldehyde crosslinking: a tool for the study of chromatin complexes. *J Biol Chem.* **2015**;290(44):26404-11.
265. **Oliver D.B., Beckwith J.** *E. coli* mutant pleiotropically defective in the export of secreted proteins. *Cell.* **1981**;25(3):765-72.
266. **Xie J., Schultz P.G.** A chemical toolkit for proteins--an expanded genetic code. *Nat Rev Mol Cell Biol.* **2006**;7(10):775-82.
267. **Farrell I.S., Toroney R., Hazen J.L., Mehl R.A., Chin J.W.** Photo-cross-linking interacting proteins with a genetically encoded benzophenone. *Nat Methods.* **2005**;2(5):377-84.
268. **Ieva R.** Site-Directed and Time-Resolved Photocrosslinking in Cells Metabolically Labeled with Radioisotopes. *Bacterial Protein Secretion Systems.* **2017**:233-45.

269. **Corning.** Pyrex Glass Code 7740 Material Properties. New York; **1987.**
270. **Tsukazaki T.** Structure-based working model of SecDF, a proton-driven bacterial protein translocation factor. *FEMS Microbiol Lett.* **2018**;365(12).
271. **Olshausen P., Defeu Soufo H., Wicker K., Heintzmann R., Graumann P., Rohrbach A.** Superresolution Imaging of Dynamic MreB Filaments in *B. subtilis*—A Multiple-Motor-Driven Transport? *Biophys J.* **2013**;105(5):1171-81.
272. **Reimold C., Defeu Soufo H.J., Dempwolff F., Graumann P.L.** Motion of Variable-Length MreB Filaments at the Bacterial Cell Membrane Influences Cell Morphology. *Molecular biology of the cell.* **2013**;24(15).
273. **Errington J.** Bacterial morphogenesis and the enigmatic MreB helix. *Nature Reviews Microbiology.* **2015**;13(4):241-8.
274. **Dunn K.W., Kamocka M.M., McDonald J.H.** A practical guide to evaluating colocalization in biological microscopy. *Am J Physiol Cell Physiol.* **2011**;300(4):C723-42.
275. **Costes S.V., Daelemans D., Cho E.H., Dobbin Z., Pavlakis G., Lockett S.** Automatic and quantitative measurement of protein-protein colocalization in live cells. *Biophys J.* **2004**;86(6):3993-4003.
276. **Liu X., Biboy J., Consoli E., Vollmer W., den Blaauwen T.** MreC and MreD balance the interaction between the elongasome proteins PBP2 and RodA. *PLoS Genet.* **2020**;16(12):e1009276.
277. **Spears K.J., Roe A.J., Gally D.L.** A comparison of enteropathogenic and enterohaemorrhagic *Escherichia coli* pathogenesis. *FEMS Microbiol Lett.* **2006**;255(2):187-202.

278. **Mellies J.L., Barron A.M.** Virulence Gene Regulation in *Escherichia coli*. *EcoSal Plus*. **2006**;2(1).
279. **Elliott S.J., Sperandio V., Giron J.A., Shin S., Mellies J.L., Wainwright L., et al.** The locus of enterocyte effacement (LEE)-encoded regulator controls expression of both LEE- and non-LEE-encoded virulence factors in enteropathogenic and enterohemorrhagic *Escherichia coli*. *Infect Immun*. **2000**;68(11):6115-26.
280. **Bustamante V.H., Santana F.J., Calva E., Puente J.L.** Transcriptional regulation of type III secretion genes in enteropathogenic *Escherichia coli*: Ler antagonizes H-NS-dependent repression. *Mol Microbiol*. **2001**;39(3):664-78.
281. **Dorman C.J.** H-NS, the genome sentinel. *Nat Rev Microbiol*. **2007**;5(2):157-61.
282. **Bustamante V.H., Villalba M.I., Garcia-Angulo V.A., Vazquez A., Martinez L.C., Jimenez R., et al.** PerC and GrlA independently regulate Ler expression in enteropathogenic *Escherichia coli*. *Mol Microbiol*. **2011**;82(2):398-415.
283. **Winardhi R.S., Gulvady R., Mellies J.L., Yan J.** Locus of enterocyte effacement-encoded regulator (Ler) of pathogenic *Escherichia coli* competes off histone-like nucleoid-structuring protein (H-NS) through noncooperative DNA binding. *J Biol Chem*. **2014**;289(20):13739-50.
284. **Platenkamp A., Mellies J.L.** Environment Controls LEE Regulation in Enteropathogenic *Escherichia coli*. *Front Microbiol*. **2018**;9:1694.
285. **Bhat A., Shin M., Jeong J.H., Kim H.J., Lim H.J., Rhee J.H., et al.** DNA looping-dependent autorepression of LEE1 P1 promoters by

Ler in enteropathogenic *Escherichia coli* (EPEC). *Proc Natl Acad Sci U S A*. **2014**;111(25):E2586-95.

286. **Bingle L.E., Constantinidou C., Shaw R.K., Islam M.S., Patel M., Snyder L.A., et al.** Microarray analysis of the Ler regulon in enteropathogenic and enterohaemorrhagic *Escherichia coli* strains. *PLoS One*. **2014**;9(1):e80160.

287. **Nakanishi N., Abe H., Ogura Y., Hayashi T., Tashiro K., Kuhara S., et al.** ppGpp with DksA controls gene expression in the locus of enterocyte effacement (LEE) pathogenicity island of enterohaemorrhagic *Escherichia coli* through activation of two virulence regulatory genes. *Mol Microbiol*. **2006**;61(1):194-205.

288. **Stein M., Kenny B., Stein M.A., Finlay B.B.** Characterization of EspC, a 110-kilodalton protein secreted by enteropathogenic *Escherichia coli* which is homologous to members of the immunoglobulin A protease-like family of secreted proteins. *J Bacteriol*. **1996**;178(22):6546-54.

289. **Berger J., Lehr H., Teitel S., Maehr H., Grunberg E.** A new antibiotic X-5108 OF *Streptomyces* origin. I. Production, isolation and properties. *J Antibiot (Tokyo)*. **1973**;26(1):15-22.

290. **Feng K., Quevedo R.E., Kohrt J.T., Oderinde M.S., Reilly U., White M.C.** Late-stage oxidative C(sp³)-H methylation. *Nature*. **2020**;580(7805):621-7.

291. **Schroeder A., Mueller O., Stocker S., Salowsky R., Leiber M., Gassmann M., et al.** The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC Mol Biol*. **2006**;7:3.

292. **Ogawara H.** Possible drugs for the treatment of bacterial infections in the future: anti-virulence drugs. *J Antibiot (Tokyo)*. **2021**;74(1):24-41.

293. **The National Institute for Health and Care Excellence.** *Clostridioides difficile* infection: antimicrobial prescribing. Public Health England; **2021**.
294. **Padavannil A., Jobichen C., Mills E., Velazquez-Campoy A., Li M., Leung K.Y., et al.** Structure of GrIR-GrIA complex that prevents GrIA activation of virulence genes. *Nat Commun.* **2013**;4:2546.
295. **Xue Y., Osborn J., Panchal A., Mellies J.L.** The RpoE Stress Response Pathway Mediates Reduction of the Virulence of Enteropathogenic *Escherichia coli* by Zinc. *Appl Environ Microbiol.* **2015**;81(11):3766-74.
296. **Bruna R.E., Casal A., Bercovich B., Gramajo H., Rodriguez E., Garcia Vescovi E.** A natural product from *Streptomyces* targets PhoP and exerts antivirulence action against *Salmonella enterica*. . **2022**;77(11):3050-63.
297. **Wang C., Liu X., Wang J., Zhou J., Cui Z., Zhang L.H.** Design and characterization of a polyamine derivative inhibiting the expression of type III secretion system in *Pseudomonas aeruginosa*. *Sci Rep.* **2016**;6:30949.
298. **Aiello D., Williams J.D., Majgier-Baranowska H., Patel I., Peet N.P., Huang J., et al.** Discovery and characterization of inhibitors of *Pseudomonas aeruginosa* type III secretion. *Antimicrob Agents Chemother.* **2010**;54(5):1988-99.
299. **Shi Y., Sun Z., Liu Y., Shu J., Zhang Y., Lv Q., et al.** Inhibition of the Type III Secretion System of *Salmonella enterica* Serovar Typhimurium via Treatment with Fraxetin. *Microbiol Spectr.* **2022**;10(6):e0294922.
300. **Tree J.J., Wang D., McInally C., Mahajan A., Layton A., Houghton I., et al.** Characterization of the effects of salicylidene

acylhydrazide compounds on type III secretion in *Escherichia coli* O157:H7. *Infect Immun.* **2009**;77(10):4209-20.

301. **Alvira S., Watkins D.W., Troman L., Allen W.J., Lorrigan J.S., Degliesposti G., et al.** Inter-membrane association of the Sec and BAM translocons for bacterial outer-membrane biogenesis. *Elife.* **2020**;9.

302. **Mamou G., Corona F., Cohen-Khait R., Housden N.G., Yeung V., Sun D., et al.** Peptidoglycan maturation controls outer membrane protein assembly. *Nature.* **2022**;606(7916):953-9.