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Characterisation of temperature dependent biofilms formed by uropathogenic *Escherichia coli* in relation to catheter associated urinary tract infections

by

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ABSTRACT

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CHARACTERISATION OF TEMPERATURE DEPENDENT BIOFILMS FORMED BY UROPATHOGENIC *ESCHERICHIA COLI* IN RELATION TO CATHETER ASSOCIATED URINARY TRACT INFECTIONS

Shannon Nicole Fenlon

Urinary tract infections (UTI) are extremely common with around half of all women experiencing at least one episode in their lifetime. Most UTIs are caused by uropathogenic *Escherichia coli* (UPEC). UTIs are the 7th leading cause of death in Singapore and are a major healthcare burden worldwide. Catheter associated UTIs (CAUTI) represent 80% of healthcare associated UTIs and are one of the few infections known definitively to be caused by biofilms. By existing in biofilms, UPEC are able to survive antibiotic treatment and evade the host immune response, complicating treatment and contributing to antibiotic resistance.

Previously, the pathogenesis of UTI and CAUTI were thought to overlap despite the latter being less extensively studied. The adhesins, type 1 pili, are essential for UPEC attachment, invasion and the subsequent formation of intracellular bacterial communities (IBCs). *In vitro* biofilm assays are commonly used as a model for attachment for both UTI and CAUTI. The standard biofilm assay, however, is performed at 25°C while the *in vivo* infection occurs at 37°C (a more physiologically relevant temperature). The aim of this thesis was to study and characterise differences in biofilm temperatures at clinically relevant temperatures, both phenotypically and genetically and to assess the relevance of this phenotype *in vivo.*

A switch in the mechanism of biofilm formation was shown at 37°C *in vitro*; biofilms grown at 25°C require type 1 pili, but those grown at 37°C do not. This type 1 pili independent biofilm formation at 37°C is shown to be relevant in an *in vivo* CAUTI model. The disparity in biofilm formation between 25°C and 37°C is relevant to the study of human CAUTI, as different sections of catheters are at different temperatures when they are in clinical use. This raises the possibility that UPEC are able to take advantage of a particular niche along the catheter and create a foothold for infection and that strains unable to cause standard uncomplicated UTI may be able to cause infection in the presence of a catheter.

In order to further characterise biofilm formation at 37°C and elucidate the mechanism behind type 1 pili independent biofilm formation, a high-throughput genomic screen was employed and revealed a potential role for several novel and previously established biofilm factors.

A clear understanding of the pathogenesis of CAUTI, and the models used to study it, will aid future investigations and the possible implementation of more effective interventions than are currently available for CAUTI.

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Declaration of Authorship

I, Shannon Nicole Fenlon, declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

Characterisation of temperature dependent biofilms formed by uropathogenic Escherichia coli in relation to catheter associated urinary tract infections

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
7. None of this work has been published before submission.

Date: 20/08/2017

Dedication

For Louise and Poppy.

Acknowledgements

I would like to take this opportunity to thank my supervisors, Dr Swaine Chen, Dr Stuart Clarke and Dr. Jeremy Webb, for their support and guidance over the course of my PhD.

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Some of the bacterial mutant strains used in this thesis were from a database of strains created by a collection of researchers in the Chen lab and some initial TraDIS data processing was carried out by Swaine Chen, both of which I'm very grateful for.

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Definitions and Abbreviations

Ag43	Antigen 43
Amp	Ampicillin
AMR	Antimicrobial resistance
ASB	Asymptomatic bacteriuria
AT	Autotransporter
BEC	Bladder epithelial cell
BHI	Brain-heart infusion
Bp	Base pair
CAUTI	Catheter associated urinary tract infection
CFU	Colony forming unit
Chlor	Chloramphenicol
CNF1	Cytotoxic necrotising factor 1
CUP	Chaperone-usher pathway
CV	Crystal violet
DEMUX	Demultiplex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
ECM	Extracellular matrix
EtOH	Ethanol
GFP	Green fluorescent protein
GIS	Genome Institute of Singapore

GU	Genitourinary
HA	Haemagglutination
HCAI	Healthcare associated infection
Hpi	Hours post infection
IBC	Intracellular bacterial communities
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IR	Inverted repeat
IRL	Inverted repeat left
IRR	Inverted repeat right
Kan	Kanamycin
Kb	Kilo-base pair
LB	Lysogeny broth
LB/amp	LB media supplemented with 100µg/ml ampicillin
LB/kan	LB media supplemented with 50µg/ml kanamycin
LOD	Limit of detection
LPS	Lipopolysaccharide
Mb	Mega-base pair
MUX	Multiplex
NGS	Next generation sequencing
nM	Nanomolar
OD	Optical density
OD_{600}	Optical density measured at a wavelength of 600nm
Pap	Pilus associated with pyelonephritis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PVC	Polyvinyl Chloride

QC	Quality control
QIR	Quiescent intracellular reservoir
Q-Q	Quantile-Quantile
SAT	Secreted autotransporter toxin
SEM	Standard error of the mean
SPATE	Serine protease autotransporters of *enterobactericeae*
Spp	Species
SSI	Surgical site infection
Tet	Tetracycline
TfR	Transferrin receptor
TraDIS	Transposon directed insertion site sequencing
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary tract infection
VAT	Vacuolating autotransporter toxin
WT	Wild type

Chapter 1: Introduction

1.1 Urinary tract infections

Urinary tract infections (UTI) are extremely common infections with around half of all women experiencing at least one episode in their lifetime (Foxman, 2002). Recurrent infections are also common in affected individuals with 24% of women experiencing a recurrence within 6 months of the first episode (Foxman et al., 2000b). Symptoms include increased frequency of urination, pain when passing urine, back pain, fever, nausea, vomiting and possibly blood in the urine. UTIs are often erroneously considered a simple nuisance, however serious consequences are possible. If left untreated UTIs can lead to significant morbidity and mortality. In 2014 UTI was the 7th highest cause of death in Singapore, accounting for 2.6% of all deaths (Ministry of Health Singapore., 2014). In the UK in 2015, diseases of the genitourinary (GU) system were the 9th highest cause of death in women, accounting for 2% of all female deaths. Included in this category are UTIs accounting for 1.17% of all female deaths (Office for National Statistics., 2015). UTIs are also a major healthcare burden; it is estimated that these community acquired infections cost the United States around 1.6 billion USD a year in antibiotic prescriptions, doctors' appointments, hospitalisation, travel, medical leave, morbidity as well as loss of output (Foxman, 2002, Foxman et al., 2008, Foxman et al., 2000a).

1.1.1 UTI classification

Numerous different types of UTI have been described and they may be classified according to multiple criteria including, but not limited to, anatomy, pathophysiology and symptoms. Anatomically UTIs may be classified as either upper or lower UTIs. A lower UTI affects the bladder and is known as cystitis, whereas an upper UTI affects the kidneys and is known as pyelonephritis. In the clinic, UTIs are often described by the pathophysiology of the urinary tract that may have caused or be caused by UTI in individual patients. For example complicated and uncomplicated UTIs; uncomplicated UTIs refer to those occurring in individuals with normal GU tract, whereas complicated UTIs occur in those with structural or functional abnormalities of the GU tract. One

major abnormality of the GU tract associated with complicated UTI is the presence of a catheter (Nicolle, 2005b).

UTIs may also be crudely classified as either symptomatic or asymptomatic. Asymptomatic cases, referred to as asymptomatic bacteriuria (ASB), occur when the urinary tract becomes infected without the manifestation of any clinical symptoms. Due to the increased focus on preventing unnecessary antibiotic use, if ASB is detected by routine testing in an otherwise healthy patient it is not recommended that it be treated with antibiotics (Nicolle et al., 2005) in order to avoid the possibility of antibiotic resistance selection.

1.1.2 Risk factors

There are a variety of risk factors that predispose to UTIs including gender, age, sexual activity, pregnancy and structural abnormalities of the urinary tract. Most UTIs are caused by opportunistic bacteria from the gut that gain access to the urinary tract via the urethral opening (Gupta and Stamm, 1999, Flores-Mireles et al., 2015, Chen et al., 2013). Therefore due to the anatomy of the urinary tract, females are at a far greater risk of contracting a UTI than males (Foxman, 2002, Marild and Jodal, 1998). Further, the urethra is much shorter in females and the urethral opening is much closer in proximity to the gastrointestinal tract facilitating the transfer of bacteria between these two sites.

Incidence of UTI also varies with age. After the age of 50, UTI incidence increases amongst men and is linked to benign prostate hyperplasia. In women, oestrogen has been shown to help prevent UTI by inducing expression of antibacterial peptides and by strengthening the uroepithelial lining as well as preventing excessive shedding of cells in response to bacterial infection (Luthje et al., 2013). This means that post-menopausal women are also at an increased risk of developing a UTI, due to the reduction in circulating oestrogen.

1.1.3 Diagnosis & Treatment

In the UK uncomplicated UTI is usually diagnosed on the presentation of clinical symptoms and positive urine culture – note should be taken however, that urine cultures are not routinely ordered for women with a suspected uncomplicated UTI and physicians often prescribe a course of empirical antibiotic based on symptoms alone. Due to the increased risks associated with complicated urinary tract infections, urine culture is recommended in order to guide treatment. When a UTI is suspected in catheterised patients, the infected catheter should be immediately removed and replaced, a urine sample obtained from the clean catheter, prior to antibiotic treatment, and finally the sample should be sent for sensitivity testing in order to guide treatment (National Institute for Health and Care Excellence., 2015, Coastal West Sussex Clinical Commissioning Group., 2013). Urine samples are taken from the clean catheter in order to hasten symptom resolution and to prevent infected catheters remaining in place, this also allows the testing of the causative bacteria in the bladder rather than those that have colonised the catheter drainage system but have not ascended to the urinary tract or are not causing symptomatic infection. Urine cultures are interpreted according to the guidelines outlined in Table 1.1 (Public Health England., 2007).

Uncomplicated UTI is generally treated empirically with a course of broad spectrum antibiotics. Commonly prescribed antibiotics for UTI's include nitrofurantoin, trimethoprim, trimethoprim/sulfamethoxazole, amoxicillin, amoxicillin/clavulanic acid (also known as co-amoxiclav or Augmentin) (National Institute for Health and Care Excellence, 2007, National Institute for Health and Care Excellence., 2015). Ciprofloxacin was previously a front line drug for UTIs but due to an increase in resistance it is now reserved for situations when other antibiotics are not available or suitable for use.

There are several alternative treatments for UTIs. One commonly discussed example is cranberry juice (and other cranberry based products). There is, however, limited data to support the use of cranberry products for the treatment of UTIs (Allan and Nicolle, 2013). A recent Cochrane review of 24 studies, including a total of 4473 participants, concluded that compared with placebo,

water, or no treatment at all, cranberry products did not reduce the occurrence of symptomatic UTI (Jepson et al., 2012). Despite these limitations, the consumption of cranberry juice for UTIs, although not recommended by clinicians, may not be discouraged as the anecdotal evidence for its positive effects outweigh any possible risk (nil if used alongside and not as a replacement for standard treatments).

Due to increasing emergence of antibiotic resistance together with the high level of recurrence of UTIs, alternatives to antibiotics for the treatment and prevention of these infections are being studied. One such example of alternative treatment options are mannosides. These are small molecule inhibitors that have been shown to be useful in mice for the prevention of bladder epithelium invasion by uropathogenic *Escherichia coli* (UPEC) via interaction with the FimH adhesin (Guiton et al., 2012).

Another key focus of research is the development of vaccines for the prevention of UTIs. Several different types of vaccines have been developed with varying degrees of success. The immunostimulant OM-89 (Uro-Vaxom®) for example, is a multi-strain (18 UPEC strains) cell lysate vaccine that has been shown to be significantly better than placebo at preventing recurrent UTI when administered both orally and vaginally (Naber et al., Brumbaugh and Mobley, 2012). Other vaccines being studied include toxin based vaccines, vaccines that target bacterial adherence, whole cell immunostimulants and cell lysate immunostimulants (Brumbaugh and Mobley, 2012, Flores-Mireles et al., 2015)

Table 1.1 Interpretation of urine cultures for UTI diagnosis
Table adapted from (Public Health England., 2007)

Type of growth	Positive interpretation
Single organism	$\geq 10^4$ colony forming units (CFU)/ml
Mixed growth (with a predominant organism*)	$\geq 10^5$ CFU/ml
E. coli or *Staphylococcus saprophyticus*	$\geq 10^3$ CFU/ml

*If three or more organisms are present with no predominance the sample is deemed to be contaminated.

1.2 Catheter associated urinary tract infections

As previously mentioned, one major class of UTIs are catheter associated urinary tract infections (CAUTI). CAUTI are a particularly big problem for healthcare systems as UTIs constitute 40% of all healthcare associated infections (HCAI) and of these 80% are catheter associated (Nicolle, 2008, Lo et al., 2008). The likelihood of contracting a UTI increases greatly with catheterisation; there is a daily infection rate of around 5% in catheterised patients (Nicolle, 2005a, Maki and Tambyah, 2001). Furthermore, CAUTIs are also particularly costly, costing the NHS on average £1122 per case (National Audit Office., 2000) and $589 per case to treat in the US (Tambyah et al., 2002). Alongside pneumonia, CAUTIs are the second most common cause of HCAIs in the UK and the US behind only GI infections in the UK (Health Protection Agency., 2012) and surgical site infections (SSI) in the US (Centers for Disease Control and Prevention (CDC). 2013).

The majority of CAUTIs are caused by Gram-negative bacteria, in particular *Escherichia coli* (Flores-Mireles et al., 2015, Kucheria et al., 2005). *E. coli* are Gram-negative rod-shaped bacteria that normally reside within the gastrointestinal tract. Most strains are harmless commensal organisms, however some are pathogenic and have the ability to cause disease (Health Protection Agency.). Other pathogens often implicated in CAUTI include *Candida* species (spp.), *Enterococcus* spp., *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Enterobacter* spp. and *Staphylococcus* spp.

These bacteria may enter the urinary tract in a variety of ways, either by contamination of the catheter upon insertion; by ascending the outside of the catheter when it is in place by capillary action; or the inner lumen of the catheter becoming colonised if either there is a break in the draining system at any point allowing bacteria to enter, or the collection bag becomes contaminated (Maki and Tambyah, 2001, Barford and Coates, 2009). CAUTIs, like UTIs, can lead to cystitis, pyelonephritis, bacteraemia and mortality.

In clinical practice the first line of treatment is to remove the catheter as soon as symptoms arise followed by antibiotic treatments to tackle any residual bacteria in the urinary tract causing symptomatic infection. As CAUTI is a biofilm-mediated infection (See Introduction section 1.4.2.3)

the majority of the causative bacteria are adhered to the surface of the catheter; removal of the catheter is therefore the fastest way to resolve the infection. The majority of cases spontaneously resolve upon the removal of the catheter (Dalen et al., 2005). This method of resolution is not always ideal for many patients, in particular those in in intensive care units (Huang, 2016) or elderly patients who require long-term catheterisation and care. Catheter removal is also not always effective in resolving CAUTI and infection recurrence is not uncommon (Jacobsen et al., 2008, Tenney and Warren, 1988). The removal procedure may also cause trauma to the urinary tract.

Scientists and healthcare professionals are continually looking for alternatives to antibiotic treatment so as to avoid contributing to the growing number of antibiotic resistant pathogens. Aside from improving basic procedure, such as better hygiene upon catheter insertion and better management of catheterised patients (Trautner, 2010, Centers for Disease Control and Prevention (CDC). 2013), there is currently a lack of evidence to support a clear strategy for the prevention and treatment of CAUTI. Good management of catheterised patients includes ensuring that catheters are only used when absolutely necessary and not as standard practice whilst further ensuring that patients requiring a catheter are only catheterised for the specific time required and no longer (Maki and Tambyah, 2001, Trautner, 2010, Centers for Disease Control and Prevention (CDC). 2013, National Clincal Guideline Centre (UK), 2012). Recent research has focused on the development of catheters that resist or prevent infection. One such example is silver coating; silver is known for its antimicrobial properties however numerous studies have shown that these silver impregnated catheters are not effective in the prevention of CAUTI (Pickard et al., 2012). Another catheter adaptation technique is antibiotic coating, which has been shown to be moderately effective in the prevention of CAUTI. Due to the concern of antibiotic resistance however, and the ever growing need to prevent it, the use of antibiotic catheters and other antibiotic prophylactic treatment is not encouraged (Tambyah, 2004).

Like UTIs, CAUTIs may be symptomatic or asymptomatic (Tambyah and Maki, 2000a, Tambyah and Maki, 2000b). The presence of a positive urine culture in asymptomatic CAUTI cases

often means that unnecessary antibiotic treatment is administered and the bacteria present in CAUTI act as a reservoir for antibiotic resistance gene selection. A recent initiative in the US aimed at reducing the number of HCAI means that hospitals are no longer reimbursed for CAUTI cases (Centers for Medicare & Medicaid Services., 2014). Although its effects are still unclear, it is thought that an increase in the number of inappropriately managed asymptomatic CAUTI cases may be observed, with increased urine testing leading to a higher rate of positive urine cultures and subsequently, increased antibiotic usage (Trautner, 2010). It is therefore essential that a clear understanding of the molecular genetics and epidemiology of CAUTI is established in order to lessen this burden and better understand how to manage symptomatic and asymptomatic cases.

1.3 UTI and CAUTI microbiology

Uropathogenic *E. coli* (UPEC) are the most common organisms implicated in both uncomplicated and complicated UTIs accounting for approximately 75% and 65% of all cases respectively (Figure 1.1) (Flores-Mireles et al., 2015). Less commonly, other bacteria may also be the cause of infection, including *Klebsiella pneumoniae, Staphylococcus saprophyticus,* and *Enterococcus* species (Figure 1.1) (Flores-Mireles et al., 2015). Multiple species may be implicated at once in CAUTI, particularly in cases of long term catheterisation (Nicolle, 2001).

1.3.1 UTI89

E. coli UTI89, the primary strain used in this thesis, is a prototypic UPEC strain isolated from a patient with cystitis (Mulvey et al., 2001b) that has been used extensively in the context of UTI research, in particular pathogenesis. UTI89 was first sequenced and described in 2006 (Chen et al., 2006) and found to have a genome size of 5.18 Mega-base pairs (Mb) encoding 5299 genes and to harbour a plasmid (pUTI89) of 114.23 Kilo-base pairs (Kb). In comparison, *E. coli* K12 (also known as MG1655), the first *E. coli* genome to be sequenced and annotated, known for being an extremely well-studied model organism, has a smaller genome consisting of 4.64 Mb encoding 4288 genes (Blattner et al., 1997).

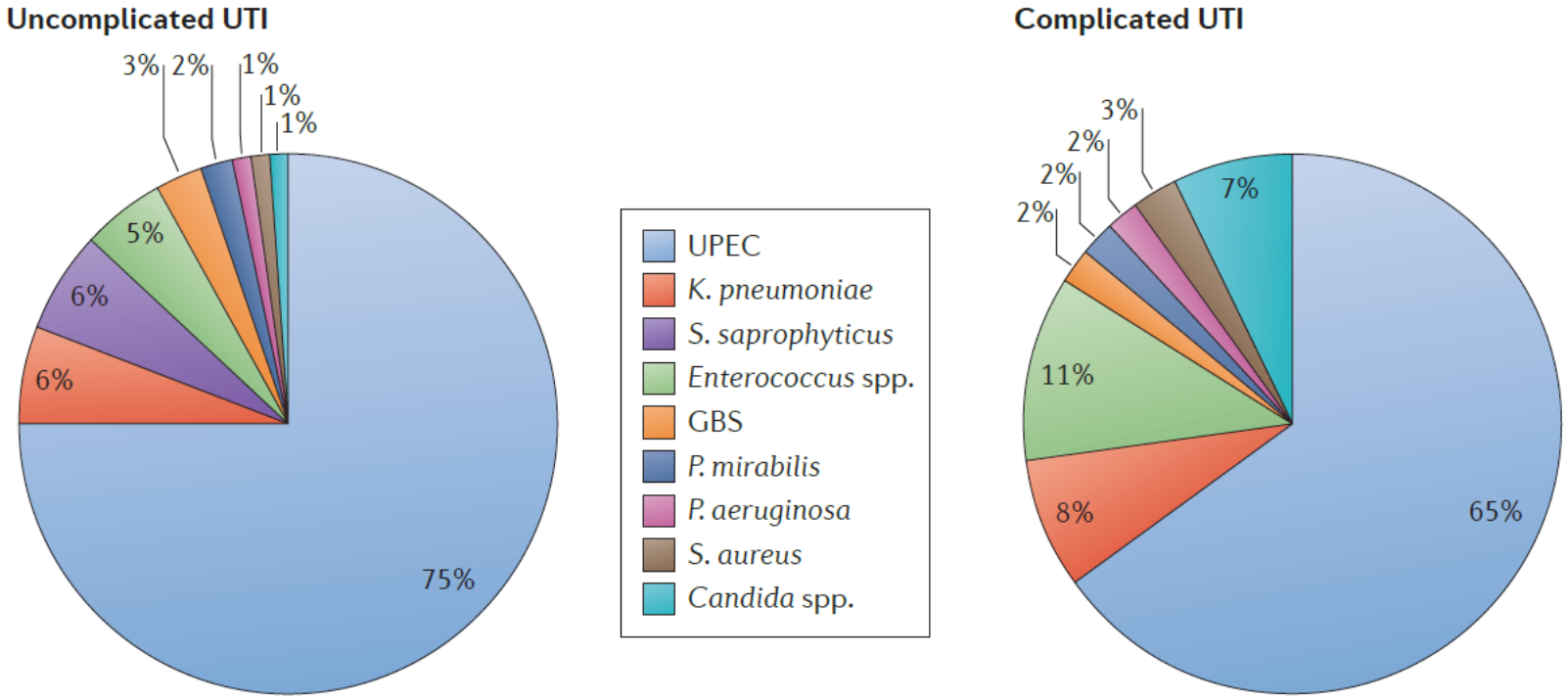

Figure 1.1 Organisms implicated in uncomplicated and complicated UTI.

Pie chart depicting the relative abundance of UPEC, *Klebsiella pneumoniae*, *Staphylococcus saprophyticus*, *Enterococcus* species, Group B *Streptococcus*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida* species.

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1.4 *E. coli* and the urinary tract

1.4.1 Virulence factors

On the most part *E. coli* is a commensal organism, however it may be able to take advantage of specific circumstances to become an opportunistic pathogen. The expression of specific virulence factors allows the cells to be pathogenic and form biofilms (see Introduction section 1.4.2) in physiological environments such as the bladder. Various different virulence factors have been attributed to UPEC pathogenesis including adhesins, flagella, iron acquisition systems, autotransporter proteins, toxins and biofilm factors (Nielubowicz and Mobley, 2010, Ulett et al., 2013).

1.4.1.1 Type 1 pili

Type 1 pili adhesins are a major virulence factor in UPEC and perhaps the best characterised example. They allow the UPEC to bind to host epithelial cells and have been shown to be essential for UTI *in vivo* (Connell et al., 1996). Type 1 pili are chaperone-usher pathway (CUP) pili encoded by a group of nine genes called the *fim* operon (Figure 1.2) (Hadjifrangiskou, 2012, Schwan, 2011).

The *fimB* and *fimE* genes encode recombinase proteins responsible for the orientation of the *fimS* invertible element. This invertible genetic element does not encode any protein but contains the promoter for the *fimA* gene; it is flanked by Inverted repeats (IRL and IRR) which allow the recombination of the contained sequence. Depending on the orientation of *fimS, fimA* expression is either switched on or off, a process known as phase variation (Figure 1.2) (Hadjifrangiskou, 2012). In turn, *fimA* encodes the major structural subuinit of the pilus. These FimA proteins form extremely stable polypeptide chains by donor strand complementation and constitute the main structure and length of the pili (Choudhury et al., 1999, Puorger et al., 2011). The *fimF* and *fimG* genes encode proteins that form a fibrillar structure and anchor the FimH element to the tip of the pilus. FimH is a D-mannose specific adhesin, responsible for the binding of UPEC to the host epithelial cells. Type 1 pili are a member of the CUP group of bacterial adhesins due to their biogenesis mechanism, *fimC*

encodes a periplasmic chaperone protein required for the transport of the pilus subunits through the periplasm to the FimD usher, a surface membrane protein responsible for the surface localisation of the pilus subunits (Schwan, 2011). Finally *fimX,* a gene located outside of the *fim* operon, elsewhere in the genome, has been shown to be involved in the regulation of the *fim* operon expression by site-specific recombination that influences the orientation of the *fimS* element contributing to phase variation (Bateman et al., Xie et al., 2006, Schwan, 2011).

1.4.1.1 Other virulence factors

1.4.1.1.1 Flagella

Flagella, bacterial cell appendages required for swimming motility, have been shown to play a minor role in UTI by aiding UPEC persistence in the GU tract and ascension into kidneys (Schwan, 2008, Lane et al., 2007, Wright et al., 2007). Flagella have also been shown to be important in *E. coli* biofilm formation, by allowing the cells to interact with abiotic surfaces and facilitating movement along them and therefore biofilm expansion (Pratt and Kolter, 1998). For this reason flagella are believed to be important for the formation and progression of CAUTI (Jacobsen et al., 2008).

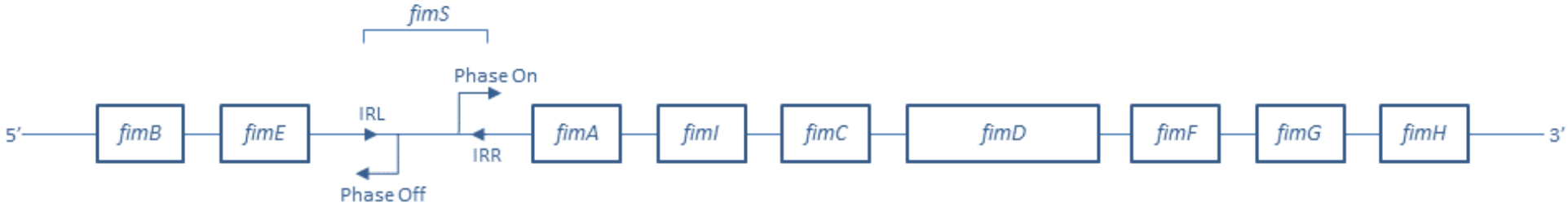


Figure 1.2 Schematic diagram illustrating the arrangement of the *fim* genes in the *fim* operon.

Figure adapted and redrawn from (Schwan, 2011). IRR denotes the right inverted repeat and IRL denotes the left inverted repeat.

1.4.1.1.2 **Toxins**

Several UPEC strains produce toxins that aid the pathogenesis of UTI. α-haemolysin, for example, is a pore forming toxin that causes the proteolysis of host cell adhesion proteins, leading to exfoliation of bladder epithelial cells (BEC). This exfoliation is thought to allow UPEC to invade the underlying epithelial cells and form quiescent intracellular reservoirs (QIR). QIRs, are small intracellular communities of bacteria, comprised of around 4-10 quiescent, non-replicating bacteria (Mysorekar and Hultgren, 2006). UPEC in these QIRs are able to resist antibiotic treatment and host immune responses and are able re-emerge and cause further infection; these are therefore believed to be the cause of recurrent and chronic infections (Mysorekar and Hultgren, 2006, Hannan et al., 2012).

Another toxin associated with UPEC is cytotoxic necrotising factor 1 (CNF1) which is encoded by 34% of UTI strains (Landraud et al., 2000). CNF1 constitutively activates Rho-GTPases that in turn leads to actin cytoskeletal rearrangement and membrane ruffling, mechanisms which are thought to aid the UPEC invasion of host cells (Lerm et al., 1999, Schmidt et al., 1997).

Vacuolating autotransporter toxin (Vat), secreted autotransporter toxin (Sat) and Pic (protease involved in intestinal colonisation) are all members of the SPATE (Serine Protease autotransporters of *Enterobactericeae*) family of proteases and are produced by UPEC. Vat has been found to be present in 68% of UPEC isolates from a collection of 45 urosepsis strains (Nichols et al., 2016). A role for both Vat and Pic in bacteraemia in mice has been identified (Subashchandrabose et al., 2013) and Vat specific antibodies have been identified in the serum of urosepsis patients indicating a role in human UTI (Nichols et al., 2016). Pic has also been shown to mediate serum resistance via an unknown mechanism in *Shigella flexneri* and *Enteroaggregative E. coli (Henderson et al., 1999)* and is also associated with UPEC, and more specifically pyelonephritis, having been identified in 31% of pyelonephritis isolates, compare to just 15% of cystitis isolates and 7% of faecal isolates (Heimer et al., 2004).

1.4.1.1.3 **Iron acquisition by UPEC**

In the human body there is a low abundance of free iron available to bacteria, as the majority is bound to proteins such as ferritin and transferrin or is part of the haem complex (Skaar, 2010). In order to survive and grow in these iron limited environments (such as the urinary tract) bacteria need to be able to sequester iron. *E. coli* has several specialised mechanisms for this purpose (Dikshit et al., 2015, Gao et al., 2012, Garcia et al., 2011). One such example of an Iron acquisition mechanism in UPEC is the recruitment of the host protein, Rab35, to endosomal compartments for intracellular survival. Rab35 is a host GTPase responsible for the recycling of transferrin receptors (TfR). UPEC recruit Rab35 and enhance its expression along with the expression of TfR increasing iron transport into the host cells, providing themselves with the means for intracellular survival (Dikshit et al., 2015) .

1.4.1.1.4 **Curli and cellulose**

Curli and cellulose are import biofilm factors for UPEC (see Introduction section 1.4.2) and therefore contribute to virulence; they have been shown to be the major components of the UTI89 extracellular matrix (ECM), constituting 85% and 15% respectively (McCrate et al., 2013). curli Curli are functional amyloid proteins (Chapman et al., 2002), which, in *E. coli,* are encoded by at least 6 genes that make up two *csg* operons, *csgBA* and *csgDEFG* (Hammar et al., 1995). Curli are important for biofilm formation and have been implicated in the initial attachment stages (Vidal et al., 1998). Curli have also been implicated in biofilm development and maturation, with curli deficient *E. coli* being unable to from complex three dimensional structures that are characteristic of mature biofilms (Kikuchi et al., 2005).

There is some overlap in the control of expression of cellulose and curli with the transcriptional activator CsgD regulating the expression of both (Brombacher et al., 2006). However increased cellulose production has been shown to negatively affect curli production and reduce biofilm formation. The role of cellulose is believed instead to be in increasing bacterial resistance to desiccation and other environmental stresses (Gualdi et al., 2008).

1.4.1.1.5 Non type 1 pili adhesins

Other than type 1 pili several other adhesins important for virulence in UPEC have been identified, including other CUP pili (see Introduction section 1.4.1.1) such as P and S pili and autotransporter (AT) adhesins such as antigen 43 (Ag43).

P pili and S pili are similar to type 1 pili in that they are also produced by a CUP mechanism, and have similar structures. They both however, have different specificities to type 1 pili. P pili, also known as Pap (pilus associated with pyelonephritis) recognise and bind Gal(α1-4)Galβ (Gal-Gal) residues found in the kidneys and are therefore implicated in upper urinary tract infections (pyelonephritis) (Johnson, 1991). S pili, similar in structure to Pap and type 1 pili, are associated with bacteria that cause neonatal meningitis and sepsis (Hacker et al., 1993, Parkkinen et al., 1988) but have been also implicated in ascending UTIs (Marre et al., 1986).

Another group of adhesins are the Dr adhesins. These adhesins play a role in cystitis and are frequently detected in UPEC from patients with lower urinary tract infections (Arthur et al., 1989). They have also been shown to interact with type IV collagen leading to persistent pyelonephritis (Selvarangan et al., 2004).

The autotransporter protein Ag43, sometimes referred to as molecular Velcro (Heras et al., 2014), is an autoaggregation factor in UPEC (Henderson et al., 1997). It contributes to biofilm formation via the mediation of cell to cell interactions. The self-recognition function of Ag43 leading to cell aggregation is well characterised, however it has also been implicated in the interaction between cells of different species leading to the formation of multi-species biofilms (Kjaergaard et al., 2000). Ag43 is also associated with enhanced resistance to antibiotics (Klemm et al., 2004).

1.4.2 Pathogenesis

1.4.2.1 Biofilms

It is thought that for the most part bacteria do not exist in their free floating planktonic form but instead biofilms - complex aggregates of cells, surrounded by an extracellular matrix (ECM).

These biofilms generally form at an interface; in this case at the bladder/urine or catheter/urine interface but, in the case of UTI, they have also been observed inside BECs (Anderson et al., 2003). The formation of biofilms is an important part of UTI and CAUTI pathogenesis.

Biofilms generally form when planktonic bacteria interact with a surface; this initial transient attachment is then superseded by stronger irreversible attachments, the biofilm then begins to mature by cell proliferation and the production of an ECM. The ECM is a complex mixture of polymeric substances that help to structure the biofilm and protect the containing cells from antibiotic treatment and host immune responses. Finally, the biofilm is then able to flux, releasing planktonic bacteria back into the environment to allow recolonization and potentially the further formation of new biofilms (Monroe, 2007).

Within the biofilm structure microcolonies have been observed, these are smaller, distinct groups of bacterial cells that exhibit increased proliferation compared to the rest of the biofilm. Bacteria within *Pseudomonas* microcolonies have been shown to be associated with a higher mutation frequency (Kostakioti et al., 2013). Biofilms are complex communities and biofilm derived variation may promote the evolution of bacteria and in turn lead to bacterial survival in the urinary tract.

Biofilm forming bacteria also have differential gene expression compared to planktonic bacteria of the same species, with studies suggesting that biofilm formation may affect the expression of up to 38% of the *E. coli* genome (Prigent-Combaret, 1999). Further, 1-15% of the genome undergoes a significant change in expression, usually corresponding to cell-cell signalling (quorum sensing) pathways (O'Toole et al., 1999, Prigent-Combaret, 1999, Van Houdt and Michiels, 2005). Cell-cell signalling has been shown to play a role in many different biofilm related processes including biofilm maturation, microcolony formation, ECM production and cell motility.

1.4.2.2 Intracellular bacterial communities (IBCs) and biofilms in UTI

A critical part of UPEC pathogenesis is the formation of intracellular bacterial communities (IBCs) (Figure 1.3). Type 1 pili are essential for the attachment to, and invasion of the bladder epithelium and subsequent formation of IBCs in mice. Type 1 pili allow the UPEC to attach to the epithelial surface in mouse bladders by binding D-mannose residues on BECs (Salit and Gotschlich, 1977b, Ofek et al., 1981). Once adhered, the FimH adhesin triggers a cascade of host protein that initiates rearrangement of the host membrane and cytoskeleton and subsequent engulfing of adhered bacteria via a zipper mechanism (Ribet and Cossart, 2015, Martinez and Hultgren, 2002). This cascade involves the phosphorylation of focal adhesion kinases (FAK) creating binding sites for PI 3-kinases which generate second messengers required for the modulation of localised host actin rearrangement (Martinez et al., 2000). Following internalisation, UPEC proliferate into communities of around 10^4 bacterial cells (Schwartz et al., 2011). These IBC structures have also been implicated directly in human UTI, having been detected in the urine of infected patients (Rosen et al., 2007), confirming the relevance of previous *in vivo* mouse data to human infection.

IBCs are believed to be intracellular biofilms, with the bacteria within them showing biofilm-like properties. They have been described as 'highly organised' and have been shown to be surrounded by an extracellular matrix. These intracellular biofilm-like structures allow UPEC to evade both host immune responses and antibiotic treatment (Anderson et al., 2003). As well as these Intracellular biofilms UPEC are also able to form microcolonies and biofilms surrounded by ECM on the bladder surface during UTI (Mulvey et al., 1998, Horsley et al., 2013).

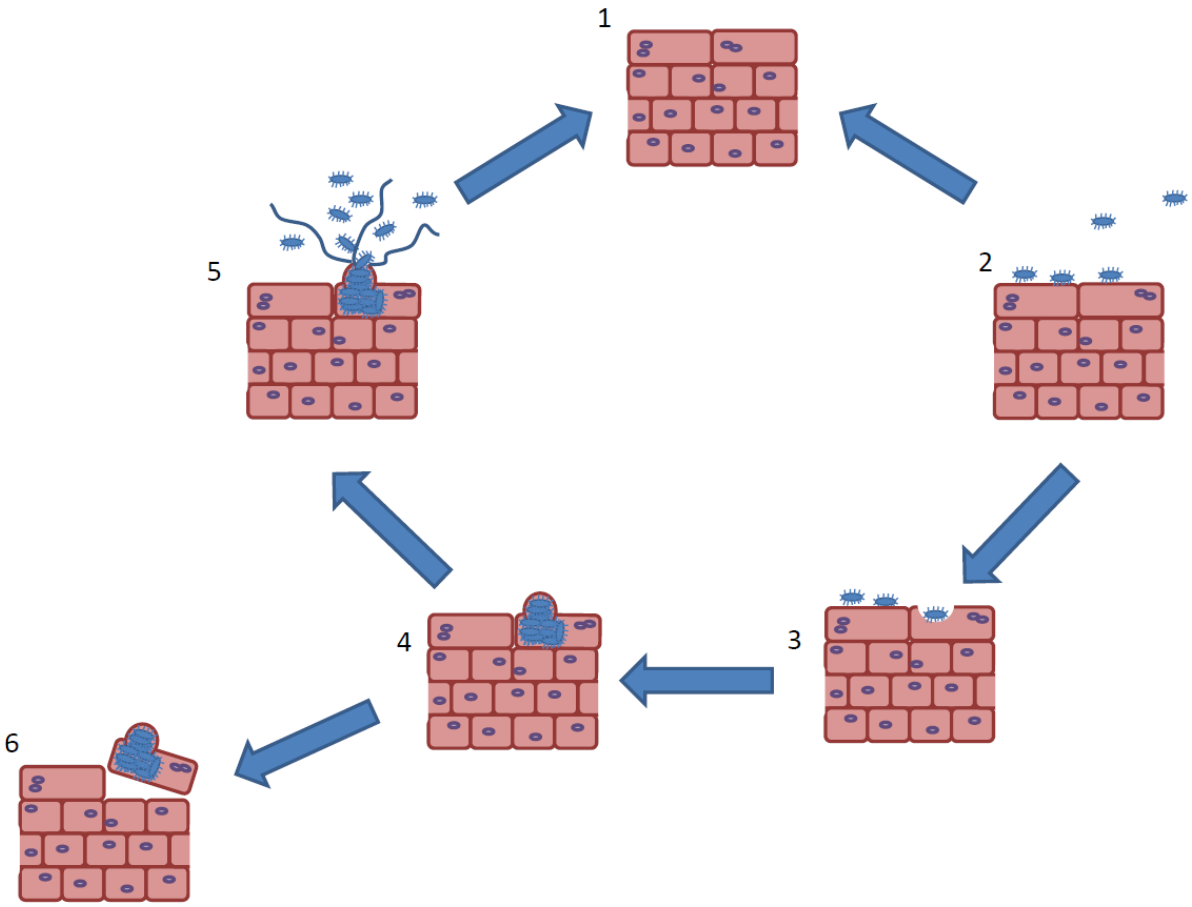


Figure 1.3 A schematic diagram of the pathogenic cycle of UPEC in UTI.

Adapted and redrawn from (Rosen et al., 2007).
1. The normal uroepithelial bladder lining. **2.** Type 1 piliated UPEC adhere to the uroepithelium via the FimH adhesin. **3.** The FimH adhesin triggers UPEC internalisation. **4.** Once inside the epithelial cell UPEC proliferate to from intracellular bacterial communities (IBCs). IBC-containing cells then either **5.** flux, reintroducing UPEC both single rod-shaped cells and filamentous UPEC back into the bladder allowing further attachment and beginning the cycle again or are **6.** exfoliated and shed in the urine.

1.4.2.3 Biofilms in CAUTI

CAUTIs are biofilm-mediated infections (Morris et al., 1999). The presence of the urinary catheter provides a favourable environment to opportunistic bacteria and provides them direct access from the periurethral area to the bladder. Upon insertion the catheter quickly becomes coated in a 'conditioning film' of host material, including collagen, fibrinogen and fibronectin and other proteins that aid bacterial binding to the catheter (Gristina, 1987, Trautner and Darouiche, 2004). This is thought to be the main reason why many attempts to generate catheters that resist or prevent bacterial colonisation have been met with limited success e.g. silver coated or antibiotic impregnated catheters (Hooton et al., 2010, Trautner and Darouiche, 2004, Pickard et al., 2012).

Once adhered the UPEC go on to develop biofilms which then mature as described in Introduction section 1.4.2.1. These mature biofilms can occlude the catheter lumen completely, blocking the drainage system and causing significant pain and distress to patients, by way of bladder distension or leakage and incontinence (Stickler, 2008, Stickler, 2014). However, because these infections are biofilm mediated, the removal of infected catheters is often the quickest and most effective way to treat them.

1.4.2.4 UPEC biofilm formation

Generally, biofilm formation is considered an extremely complex process involving many different genes and their differential regulation and expression; in UPEC it is still not fully understood. As previously mentioned, expression of as much as 38% of the *E. coli* genome is affected during biofilm formation (Prigent-Combaret, 1999). Several studies have identified potential *E. coli* biofilm factors at the expression level using techniques such as microarrays and macroarrays, with many of the genes identified in these studies being novel with previously unknown functions (Beloin et al., 2004, Ren et al., 2004, Schembri et al., 2003).

Aside from expression analyses, several different approaches to genetic studies have also identified a series of factors that may be involved in *E. coli* biofilm formation. In one such study Niba et al. (2007) used a collection of 3985 systematically created, in frame deletion mutants of non-

essential genes in the laboratory *E. coli* strain K-12. This collection of mutants is known as the Keio collection (Baba et al., 2006b). By studying the biofilm formation of these mutant strains *in vitro* these researchers were able to identify 110 genes potentially involved in biofilm formation.

More specifically, UPEC biofilm formation also appears to be very complex with hundreds of genes having been implicated. Hadjifrangiskou et al. (2012) for example, used a traditional transposon mutagenesis screen in order to identify genes involved in UPEC (UTI89) biofilm formation. Using this method they were able to screen 6144 UTI89 transposon mutants in several different biofilm conditions and identified 170 mutants which displayed reduced biofilm formation abilities.

These genome wide approaches have confirmed the importance of well characterised examples of virulence and biofilm factors such as type 1 pili, curli, cellulose, flagella and Antigen 43 (as described in 1.4.1), however they also identify a role for many novel, previously uncharacterised genes in biofilm formation including several involved in lipopolysaccharide (LPS) synthesis. LPS synthesis has been shown in several studies to play a role in biofilm formation although its exact role is not fully understood. Two possible mechanisms of LPS effects on biofilm formation have been described, direct effects whereby the LPS itself is involved directly in adhesion and/or biofilm formation and indirect effects, where LPS mutation affect other biofilm factors and in turn affect biofilm formation (Beloin et al., 2006). Furthermore, a recent study suggested that some LPS mutants resulted in enhanced biofilm phenotypes however the converse has also been observed (Chalabaev et al., 2014, Nakao et al., 2012) This information combined shows how complex LPS involvement in biofilm formation may be. A specific example of LPS involvement in biofilm formation has been described by Chalabaev et al. (2014). They observed increased palmitoylation of the Lipid A component of LPS in *E.coli* biofilms. This is thought to contribute to biofilm tolerance to host immune responses (Chalabaev et al., 2014). A similar principle has also been described by Aguiniga et al. (2016) where an LPS mutant, generated by deletion of an O-antigen ligase (*waaL*),

displayed increased neutrophil recruitment, suggesting that LPS components are able to modulate host immune responses.

1.4.2.5 Biofilm mediated antibiotic resistance in UTI and CAUTI

Bacteria existing in biofilms have been shown to be between ten and a thousand times more resistant to antimicrobial substances than their planktonic counterparts (Davies, 2003, Luppens et al., 2002). Biofilms confer this resistance in a number of ways – one of these ways, oxygen limitation, occurs in cells within oxygen limited areas of the biofilm that are metabolically inactive, rendering them resistant to antibiotics (Walters et al., 2003). Nutrient gradients within the biofilm also contribute to the decreased metabolic activity of the innermost cells and therefore antibiotic resistance. Cells within biofilms also show differential expression to their planktonic counterparts and often express protective, such as multidrug efflux pumps, contributing to antibiotic resistance (Fux et al., 2005, Stewart, 2002, Davies, 2003).

Biofilms form on catheters, the bladder surface and intracellularly as IBCs; the physical structure (and intracellular nature in the case of IBCs) of these biofilm aggregates mean that antibiotic treatment is particularly problematic. Antibiotic efficacy is further reduced by the surrounding protective matrix which helps to prevent antibiotic penetration. Therefore, the antibiotic can only reach the outermost bacteria in the structure (Davies, 2003, Luppens et al., 2002), or not reach the bacteria at all, in the case of IBCs (Mulvey et al., 2001a). This potentially allows the selection of antibiotic resistance genes as well as leaving a reservoir of bacteria unharmed within the biofilm that are capable of causing subsequent and recurrent infection.

The spatial arrangement of cells within biofilms is thought to influence antibiotic resistance in biofilms. Increased horizontal transfer of antibacterial resistance genes has been observed in biofilms (Savage et al., 2013, Krol et al., 2011, Molin and Tolker-Nielsen, 2003) and is believed to be aided by the close proximity of cells to each other (Savage et al., 2013). Mutation rates are higher in biofilms compared to planktonic growth. This increased mutability allows cells within biofilms may also accumulate mutations that may lead to antibiotic resistance and favour survival within

biofilms (Hoiby et al., 2010, Driffield et al., 2008). For these reasons, as previously mentioned, the current recommended treatment of CAUTI is removal of the infected catheter. The removal of the catheter often resolves these biofilm-mediated infections immediately, however it may be followed up with antibiotic treatment if necessary in symptomatic cases. However, this course of action is not always ideal and the infection may recur. Antibiotic treatment of these infections contributes to the emergence of antibiotic resistance and CAUTIs are considered to be possibly the largest reservoir for antimicrobial resistance (AMR) in hospitals (Tambyah and Maki, 2000a, Schaberg et al., 1976).

1.5 Modelling UTI and CAUTI

1.5.1 *In vitro* biofilm models of CAUTI and UTI

As described in Introduction section 1.4.2, CAUTI and UTI are known to be biofilm-mediated infections. There are various models available for the *in vivo* study of these biofilms, namely flow cells (Christensen et al., 1999), microtitre plate assays (O'Toole, 2011), colony biofilms (Ray et al., 2012) and pellicle biofilms (Hung et al., 2013) as well as animal models. Flow cell models allow the study of biofilms under semi-realistic conditions and are generally considered the gold standard in the study of biofilms (Christensen et al., 1999, Branda et al., 2005). These flow cell models have been adapted by various groups in order to better apply to certain types of biofilm formation. For example Stickler et al. (1999) developed a 'bladder' model that better mimics the flow of urine in the urinary tract and allows the study of catheter biofilm formation in a more realistic setting (Stickler et al., 1999, Reisner et al., 2014).

Microtitre plate assays are one of the more commonly utilised as they are simple, versatile and extremely cost effective due to their high-throughput nature (O'Toole, 2011). In general biofilms are allowed to form in a microtitre plates are subsequently stained in order to quantify biofilm formation. However, the conditions under which biofilms are formed in a microtitre plate are very different to natural conditions; it is well known that small changes in growth conditions

can greatly affect biofilm formation (Moreira et al., 2013, Stepanović et al., 2007, Naves et al., 2008). Many studies have utilised this CV microtitre plate assay in order to test the biofilm forming ability of clinical strains and mutants and model type 1 pili mediated biofilm formation. However the exact conditions applied to the assay have been known to vary from application to application. Even within the field of UPEC there is variation, usually depending on the virulence factor being studied. For example, this assay is often used to model type 1 pili mediated biofilm formation, for this purpose it is mostly used at a temperature of 25°C in Lysogeny Broth (LB), although not always (Rosen et al., 2008, Agarwal et al., 2013, Lo et al., 2014, Hadjifrangiskou et al., 2012, Niba et al., 2007, Pratt and Kolter, 1998). This variation in published assays, outlined in Table 1.2, complicates the interpretation of these biofilm assays within the UTI field, as it has been shown that variations in test conditions can lead to variation in biofilm phenotypes. Using a transposon screening approach researchers have observed differences in the genes involved in several variations of *in vitro* biofilm models (Hadjifrangiskou et al., 2012), suggesting that there are different biofilm mechanisms involved in different models.

1.5.2 Animal models of UTI and CAUTI

Several very effective murine models have been established and well characterised for the study of both UTI and CAUTI. For UTI there are various different animal models for the study of various aspects of uncomplicated UTIs. Mice are the most commonly used animals however there are also other animal models, including Zebrafish (Wiles et al., 2009). In 2009 Hung et al developed a standardised model for UTI, based on a previous mouse model (Hung et al., 2009, Hopkins et al., 1995), that has been well utilised to study the pathogenesis of UTI. In this model, mice (typically C575BL/6 or C3H/HeN mice) are transurethrally infected with an inoculum of 2×10^7 CFU/50 µl. The length of the needle used ensures the inoculum is deposited directly into the bladder, while the inoculum size has been sufficiently optimised to avoid both vesicoureteral reflux and exfoliation of the bladder epithelium (seen often with higher bacterial loads); slow administration of the inoculum also helps to prevent vesicoureteral reflux. Infection in this mouse model, and similar

models with inoculums of higher bacterial load, has been clearly shown to be dependent on type pili (Connell et al., 1996, Wright et al., 2007).

The CAUTI murine model, optimised by Guiton et al. (2010) and fully described by Conover et al. (2015) is an adaptation of the UTI model previously described, and has been used to study *Enterococcus faecalis* infection. C57BL/6 mice are used due to their ability to retain catheter implants in their bladders (Conover et al., 2015). C3H/HeN mice, also commonly used for UTI infections, do not retain the catheter implants as mentioned by P. Guiton and referenced as a personal communication by Conover et al. (2015). Prior to inoculation, a 5mm silicone tube implant is inserted directly into the bladder via the urethra. The inoculation is then performed as in the standard UTI model.

1.5.3 Limitation of CAUTI models

As discussed in the previous section, several laboratory assays exist to model biofilm formation *in vitro* including the CV microtitre plate assay. The CV assay, although not necessarily the best model for natural conditions, is very commonly used due to its ease and high throughput nature; however the conditions used for published CV assays vary in their incubation times and temperatures as well as the growth media and adherence material used. UPEC Biofilm formation in this assay has however been shown to be type 1 pili dependent at 25°C, when grown in LB media and using PVC plates. This corresponds to strong *in vivo* data from a mouse model of UTI, showing UTI to be dependent on type 1 pili. For CAUTI however, the correspondence between models is less clear. *In vitro* modelling is complicated by the variation in temperature of a catheter when in clinical use (from 25°C to 37°C) alongside the variation in adherence material. Silicone catheters are commonly used in the clinical setting but silicone microtitre plates are not available for use in the *in vitro* CV assay. This makes it difficult to relate *in vitro* findings to a murine CAUTI model that uses silicone implants to study abiotic attachment in *vivo.*

Table 1.2 Published biofilm assays and their conditions

A selection of published CV microtitre biofilm assays demonstrating the variation in conditions (incubation temperature, media and length of incubation) between experiments and specific biofilm factors being studied.

Biofilm factor being studied	Conditions			Reference
	Incubation temperature	Growth media	Incubation time	
Type 1 pili	Room temperature	LB	48 hours	Hadjifrangiskou et al. (2012)
Type 1 pili and general biofilm formation of UPEC mutant library	Room temperature	LB	48 hours	Lo et al. (2014)
Type 1 pili in *Klebsiella pneumonia*	Room temperature	LB	48 hours	Rosen et al. (2008)
Type 1 pili	30°C	M9	24 hours	Agarwal et al. (2013)
General biofilm formation of clinical *E. coli* isolates	28°C	LB	24 hours	Melican et al. (2011)
Type 1 pili	37°C	M63B1-Gluc	24 hours	Khanal et al. (2015)
Type 1 pili	30°C	M63	24 hours	Soto et al. (2007)
Type 1 pili and other biofilm factors	25°C	LB	24 and 48 hours	Pratt and Kolter (1998)

1.6 Aims, objectives and hypotheses

UTI and CAUTI, caused predominantly by UPEC, are major healthcare burdens both medically and financially; they are expensive to treat, cause increased length of hospital stays, result in morbidity and mortality and are a major reservoir for AMR. A clear understanding of the pathogenesis of both UTI and CAUTI is therefore vital to inform research into effective prevention and treatment strategies (Flores-Mireles et al., 2015, Tambyah and Maki, 2000a, Foxman, 2002).

It is clear that biofilms play important roles in both CAUTI and UTI. In UTI, biofilms form intracellularly as IBCs as well as in aggregates on the bladder surface (Rosen et al., 2007, Mulvey et al., 1998, Anderson et al., 2003), whilst in CAUTI, substantial biofilms form on the catheter surface (Morris et al., 1999, Stickler, 2008). Type 1 pili, a major virulence factor for UPEC, are essential for UTI and have been implicated in biofilm formation. However, UPEC pathogenesis in CAUTI is less well understood (Jacobsen et al., 2008). As type 1 pili are vital for UTI pathogenesis and have been implicated in biofilm formation, it is thought that they may also play a role in CAUTI (Mobley et al., 1987). However, an initial observation in the Chen lab (Majid Eshaghi & Swaine Chen, personal communication, September 2013) suggested that biofilm formation by UTI89 did not require type 1 pili at 37°C.

The potential ability of UTI89 to change its biofilm formation phenotype at various temperatures is particularly interesting in the context of CAUTIs. When in clinical use, catheters are maintained at varying temperatures along their length: from the collection bag at room temperature, to the middle portion of the tubing which is usually in close contact to the patients skin (though still external to the urinary tract), and finally to the tip and initial tubing which lie in the urinary tract (bladder and urethra) at body temperature (37°C). The observed phenotype raises the possibility that UPEC may be able take advantage of a particular niche along the length of the catheter in order to create a foothold for infection. Strains that are unable to cause infection in a healthy bladder may be able to cause infection in the presence of a catheter.

The major aim of this project is to investigate and characterise the previously observed difference in the biofilm formation of UTI89 type 1 pili mutants at varying temperatures, in order to address the question of whether biofilm formation is type 1 pili dependent at 37°C, a more physiologically relevant temperature and to assess the relevance of this phenotype to CAUTI *in vivo*. By doing so, this thesis also aims to generate a better understanding of an *in vitro* biofilm model (CV assay) in relation to CAUTI and to elucidate any correspondence between this *in vitro* model and *in vivo* data from a mouse CAUTI model. These aims will be achieved through the utilisation of various different techniques and approaches including *in vitro* phenotypic and genetic characterisation, *in vivo* characterisation using UTI and CAUTI murine models and finally, the characterisation of UPEC isolates obtained from CAUTI patients.

1.6.1 Hypotheses

The overall research hypotheses for this thesis are as follows:

1. CAUTI pathogenesis differs to that of UTI and is not dependent on type 1 pili; despite being essential for UTI pathogenesis, type 1 pili are not required for CAUTI.
2. At 37°C the *in vitro* model for biofilm formation (CV assay) corresponds to the *in vivo* mouse CAUTI model:
    - Biofilm formation on PVC in an *in vitro* model of biofilm formation (CV assay) is type 1 pili independent at 37°C.
    - Type 1 pili are not required for CAUTI *in vivo.* UTI89 can cause infection and colonise catheter implants, in a mouse CAUTI model, in the absence of type 1 pili.
    - Pyelonephritis strain CFT073 is also able to cause infection and colonise catheters in a mouse model for CAUTI

1.6.2 Specific aims and objectives by chapter

The specific aims of each chapter in this thesis are outlined below:

Chapter 3: Genetic Characterisation of a Temperature Dependent Biofilm

Chapter 3 aims to validate and characterise the initial observation that biofilm formation by UTI89 varies with temperature (Majid Eshaghi & Swaine Chen, personal communication, September 2013) and to verify this phenotype using different attachment surfaces, genetic mutants and chemical inhibition studies.

Chapter 4: *In Vivo* Characterisation of Type 1 Pili Independent Biofilm Formation

Chapter 4 aims to assess the *in vivo* relevance of varying biofilm formation mechanisms by testing the ability of type 1 pili deficient UTI89 to cause infection *in vivo,* in a murine model for CAUTI, and to elucidate any correspondence between biofilm formation *in vitro* and the *in vivo* CAUTI model.

Pyelonephritis strain CFT073 which is also able to cause infection and colonise catheters in a mouse model for CAUTI

Chapter 1:

Genomic Approaches to Biofilm Phenotype Characterisation

The aim of chapter 5 is to genetically characterise the variation in biofilm formation mechanism at different temperatures. This work is currently in progress; transposon directed insertion site sequencing (TraDIS) will be utilised with a view to identifying causative genes responsible for biofilm formation *in vitro* at both 25°C and 37°C.

Chapter 2: Materials and Methods

2.1 Bacterial strains

The majority of the mutant strains used in this thesis are part of an existing database created by researchers in the Chen laboratory (where the research in this thesis was performed). All strains used and created for this thesis are detailed in Table 2.1; those marked with an asterisk were created by Shannon Fenlon specifically for this thesis (see Materials and Methods sections 2.1.3, 2.1.4 and 2.1.5 for construction details). For clarity, Table 2.1 also details the official strain names as well as the descriptive strain names used in the figures in the later results chapters of this thesis.

2.1.1 Bacterial culture

All strains were cultured in LB media at 37°C, unless otherwise stated, supplemented with the appropriate antibiotic listed in Table 2.1 where required.

Table 2.1 Description of the bacterial strains and the relative antibiotics and concentrations used.

* Strains created by Shannon Fenlon specifically for this thesis (for construction details see Materials and Methods sections 2.1.3, 2.1.4 and 2.1.5)

Strain	Detailed description	Name used in figures	Resistance marker & antibiotic concentration used for selection
SLC-6	UTI89 wild type (WT)	UTI89	-
SLC-7	MG1655 wild type	MG1655	-
SLC-66	UTI89 -WT FimH reintegrated Complement for Q133K mutant. SLC-67	Q133K complement	Kanamycin 50 µg/ml
SLC-67	UTI89 *fim*H Q133K	Q133K	Kanamycin 50 µg/ml
SLC-79	UTI89 *fim*H A62S	A62S	Kanamycin 50 µg/ml
SLC-152	CFT073Δ*fim*H	CFT073Δ*fim*H	Chloramphenicol 20 µg/ml
SLC-153	MG1655Δ*fim*H	MG1655Δ*fim*H	Chloramphenicol 20 µg/ml
SLC-282	UTI89Δ*fim* (full *fim* operon knockout)	UTI89Δ*fim*	-
SLC-295	UTI89/pANT4 (UTI89 constitutively expressing GFP)		Kanamycin (Plasmid) 50 µg/ml Ampicillin (Plasmid) 100 µg/ml
SLC-309	CFT073 wild type	CFT073	-
SLC-443	UTI89Δ*csg*G-C (full curli (*csg)* operon knockout)	UTI89Δ*csg*	Kanamycin 50 µg/ml

Strain	Detailed description	Name used in figures	Resistance marker & antibiotic concentration used for selection
SLC-490	UTI89 Phase locked ON (Inverted repeat (IR) mutant)	Phase ON	-
SLC-492	UTI89 Phase locked OFF (IR mutant)	Phase OFF	-
SLC-747	UTI89Δ*fli*C	UTI89Δ*fli*C	Kanamycin 50 µg/ml
SLC-911	UTI89 plasmid complementation of *fim* operon	*fim* complement	Tetracycline (Plasmid) 10 µg/ml
E. faecalis (OG1RF)	*Enterococcus faecalis* (strain OG1RF)	*E. faecalis*	Cultured in Brain Heart Infusion (BHI) media
SLC-912* (SNF1-51-1)	UTI89Δ*fim*/pANT4 (UTI89Δ*fim* constitutively expressing GFP)		Kanamycin (Plasmid) 50 µg/ml Ampicillin (Plasmid) 100 µg/ml
SLC-914* (SNF1-48-9)	SLC-282Δ*csg*G-C (knockout of both *fim* and *csg* operons)	UTI89Δ*fim*Δ*csg*	Kanamycin 50 µg/ml
KSM5-64-5	UTI89Δ*sfa* full *sfa* (S pilus) operon Knockout	UTI89Δ*sfa*	Kanamycin 50 µg/ml

2.1.2 Growth curves

Sequential optical density (OD) measurements were taken to generate planktonic growth curves prior to biofilm formation in order to assess the growth patterns of the *E. coli* strains used, with a view to detecting any growth defects or advantages that may have been brought about during genetic manipulation. Growth curves were obtained at both 25°C and 37°C using the 'Bioscreen C' instrument from Oy Growth Curves Ab Ltd. A single colony was used to inoculate 2 ml of LB (supplemented with antibiotics where appropriate - Table 2.1) and incubated overnight at 37°C in a shaking incubator. Cultures were then adjusted to an OD_{600} (optical density measured at a wavelength of 600 nm) of 1 and diluted 100 fold in a final volume of 150 µl in wells of a Bioscreen honeycomb plate (Oy Growth Curves Ab Ltd.). The Bioscreen machine was set to take OD (Optical density) measurements at a wavelength of 600 nm every 20 minutes for 24 hours, shaking the cultures very briefly 10 seconds prior to each measurement.

2.1.3 Generating electrocompetent cells

Electrocompetent cells required for genetic manipulation and transformation were generated as follows: Firstly the required strain was grown up in 2 ml liquid culture (LB) from single colonies overnight in a shaking incubator at 37°C (or 30°C for cells harbouring pKM208, see Materials and Methods section 2.1.4). Cells were subcultured by a 1:100 dilution into 50 ml LB, in a 250ml Erlenmeyer flask, and grown in a shaking incubator at 37°C (or at 30°C for cells harbouring pKM208), until they reached an OD_{600} of 0.55-0.6 and then transferred to falcon tubes in two 50 ml aliquots and placed on ice (the rest of the procedure was carried out on ice, using a centrifuge cooled to 4°C and using reagents refrigerated to 4°C). The cells were centrifuged at 5000 rpm for 10 minutes using a benchtop centrifuge (Eppendorf 5804R), the supernatant was then carefully poured off and the cells resuspended in 50 ml sterile cold water. This procedure was repeated so the cells were washed twice in water, and after the second wash the cells were resuspended in 1

ml cold 10% glycerol and centrifuged for 10 minutes at 5600 rpm in a 1.5 ml microcentrifuge tube using a benchtop centrifuge (Eppendorf 5430). Finally, the cells were resuspended in 150 µl glycerol (10% v/v) and spilt into three 50 µl aliquots, flash frozen in liquid nitrogen and stored at -80°C.

2.1.4 Generation of UTI89 double *fim* and *csg* operon knockout strain

SLC-914 is a double knockout of the type 1 pili (*fim*) and the curli (*csg*) operons from UTI89 (UTI89Δ*fim*Δ*csg)*. It was generated by λ-red recombinase mediated knockout of the *csg* operon (homologous recombination) from SLC-282 (UTI89Δ*fim*) in order to test the dependence of biofilm formation on type 1 pili and curli. Briefly, UTI89Δ*fim* cells (SLC-282) were made electrocompetent, as described in Materials and Methods section 2.1.3, and then transformed with pKM208 (Murphy and Campellone, 2003) (commercially available from Addgene). The plasmid pKM208 harbours the genes required for efficient recombination and is present in a low copy number and under the control of an IPTG (Isopropyl β-D-1-thiogalactopyranoside) inducible promoter. Its origin of replication is temperature sensitive and the plasmid is therefore maintained at 30°C. This was carried out as detailed below:

Transformation of pKM208 into SLC-282 cells was performed using a 50 µl aliquot of electrocompetent SLC-282 cells, generated according to the protocol in Materials and Methods section 2.1.3. SLC-282 cells were thawed on ice and 1 µg of plasmid DNA (pKM208) was added to the cells and mixed by careful pipetting up and down. The mixture was then transferred to a 1 mm electroporation cuvette (BioRad) and electroporated using the BioRad Gene Pulser Xcell™ machine at the following settings: 1.7 kV, 25 µF, 400 Ω. Following electroporation, the cells were immediately resuspended in 1 ml of pre warmed (30°C) LB media and allowed to recover for 2 hours in a shaking incubator at 30°C. Post-recovery, a 50 µl aliquot of the transformed cells was spread onto a LB plate supplemented with 100 µg/ml ampicillin (amp), referred to as LB/amp from here onwards, and incubated at 30°C overnight to select for cells successfully transformed with pKM208.

A single pKM208 positive cell (amp resistant) was selected and used to inoculate 2 ml LB/amp and incubated at 30°C in a shaking incubator until turbid. A 900 µl aliquot of this turbid culture was mixed with 100 µl of the cryoprotectant dimethyl sulfoxide (DMSO) and stored at -80°C. This stored UTI89Δ*fim* strain harbouring pKM208 was named SNF1-47-1 (See Table 2.1)

The expression of the red recombinase proteins, required for efficient homologous recombination, was then induced by IPTG in the SNF1-47-1 cells. Briefly, a 2 ml overnight culture of SNF1-47-1 was started from the frozen stock (in LB/amp) and incubated at 30°C in a shaking incubator. SNF1-47-1 cells were subcultured by a 1:100 dilution into 15 ml LB/amp and incubated at 30°C in a shaking incubator until they grew to an OD_{600} of 0.3-0.4. IPTG was then added to a concentration of 1 mM and incubated for a further 30 minutes, in order to induce the expression of red recombinase from the pKM208 plasmid. The cells were subjected to heat shock in a water bath at 42°C, and mixed by swirling frequently and placed on ice for 15 minutes, as this has been shown to increase the recombination rate (Murphy and Campellone, 2003), again agitating regularly. In order to halt the IPTG induction of Red recombinase. The induced cells were made electrocompetent by centrifugation using a benchtop centrifuge (Eppendorf 5804R) for 10 minutes at 5000 rpm followed by removal of the supernatant and resuspension of the cells in cold sterile water; this was repeated for a total of 2 washes in sterile water. The cells were then resuspended in 1 ml 10% glycerol and centrifuged for 5 minutes at 5000 rpm, again the supernatant was removed however this time the cells were resuspended in 50 µl 10% glycerol. The resulting cell suspension (competent SLC-282 cells expressing red recombinase) was kept on ice until the subsequent electroporation with the appropriate polymerise chain reaction (PCR) product to knock out the *csg* operon as detailed below:

Primers were designed to amplify the kanamycin (kan) cassette from the commercially available plasmid pKD4 (Addgene) (Datsenko and Wanner, 2000) with 50 bp (base pair) homology to the 5’ and 3’ end of the *csg* operon (see Table 2.2). The PCR reaction was set up, using the primers

detailed in Table 2.2 and the reaction micture was set up as follows: 35 µl water, 10 µl 5X Phusion® HF buffer (New England BioLabs), 1 µl deoxyribonucleotide triphosphate (dNTP), 1.25 µl forward knockout pimer (Table 2.2), 1.25 µl reverse knockout primer (Table 2.2), 0.5 µl Phusion® High-Fidelity DNA polymerase and 1 µl pkD4 template.

The resulting PCR product was cleaned up by passing through an Amicon Ultra 0.5 ml centrifugal filter (according to the manufacturer's protocol): 1 µg of this clean PCR product was added to the competent IPTG induced SLC-282 cells and carefully mixed by pipetting. The cell/DNA mixture was electroporated in a 1 mm electroporation cuvette using the Gene Pulser Xcell™ machine (both BioRad) at the following settings: 1.5 kV, 25 µF, 400 Ω.

The electroporated cells were immediately resuspended in 1 ml pre-warmed LB (37°C) and allowed to recover for 2 hours in a shaking incubator at 37°C followed by further 2 hours incubation at 37°C in a static incubator. The recovered cells were spread on to LB agar supplemented with 50 µg/ml kanamycin (LB/kan) and incubated overnight at 37°C; the pKM208 plasmid is not be maintained at this temperature and was be lost from the population during this incubation. Colonies were then screened by PCR, using the test primers detailed in Table 2.2. These primers are for the outside sequence flanking the *csg* operon at both the 5' and 3' ends. Colony PCR was carried out as follows: Firstly, the PCR reaction mixture was set up using the test primers detailed in Table 2.2 as follows: 41.5 µl water, 5 µl 10X ThermoPol buffer (New England BioLabs), 1 µl dNTP, 1 µl forward test primer, 1 µl reverse test primer and 0.5 µl *Taq* DNA polymerase. Individual colonies were collected using a 1 µl inoculation loop, and transferred onto LB/kan agar plates by lightly touching the agar. The same inoculation loop was then used to transfer the remaining cells directly into the PCR reaction mixture.

Using a clean inoculation loop the cells previously transferred onto LB/kan were steaked to single colonies and incubated overnight at 37°C. The resulting PCR products were visualised by gel electrophoresis, using a 1.5% agarose gel. Unsuccessful recombination resulted in a PCR product

the size of the *csg* operon (as it was still present and amplified during the PCR reaction). Successful recombination, however, was reflected by a smaller PCR product of around 2 kb (the size of the kan cassette used to replace the *csg* operon by homologous recombination). A PCR positive knockout colony was used to inoculate 2ml LB/kan and grown up until turbid. A 900 µl aliquot of this cell suspension was mixed with 100 µl DMSO and stored at -80°C. This resulting strain, UTI89Δ*fim*Δ*csg*, was named SLC-914 (Table 2.1).

Table 2.2 Primers used to knock the *csg* operon out of SLC-282 and test for successful recombination.

Primer Type	Direction	Sequence (5'-3')
Knockout	Forward	TTCGCTTAAACAGTAAAATGCCGGATTATTAATTCCGGCATTTTTATCTAGTGTGTAGGCTGGAGCTGCTTC
Knockout	Reverse	GCAGCAGACCATTCTCTCCAGGTTCATCTTATGCTCGATATTTGCAAVAAACATATGAATATCCTCCTTAG
Test	Forward	GCGCTCGGACTGGTATTTGG
Test	Reverse	GTCACATGCCGGTTCCACAA

2.1.5 Generation of GFP expressing UTI89Δ*fim* strain

SLC-912 is a UTI89Δ*fim* strain that constitutively expresses green fluorescent protein (GFP). It was generated by transformation of the GFP reporter plasmid pANT4 (previously known as pComGFP) (Cormack et al., 1996) into host strain SLC-282 (UTI89Δ*fim*). SLC-282 cells were made electrocompetent as described in Materials and Methods section 2.1.2. A frozen aliquot of these competent cells was thawed on ice and 100 ng of pANT4 was added and mixed by pipetting. The cell/DNA mixture was transferred to a 1 mm electroporation cuvette and electroporated using a Gene pulser Xcell™ machine (both from BioRad) at the following settings: 1.7 kV, 25 µF, 400 Ω. Post-electroporation, the cells were immediately resuspended in 1 ml pre-warmed LB (37°C) and the cells allowed to recover by incubation at 37°C shaking. The cells were then spread onto LB/kan agar (as pANT4 also contains a kan resistance cassette) and incubated overnight at 37°C. The colonies were visualized using an Olympus MVX10 fluorescence microscope to check for GFP expression and a GFP positive colony was selected for storage. The selected colony was grown up in 2ml LB/kan at 37°C until turbid; a 900µl aliquot of this cell suspension was then mixed with 100 µl DMSO and stored at -80°C. This resulting strain was named SLC-912 (Table 2.1).

2.2 Biofilm assays

2.2.1 Microtitre plate crystal violet (CV) biofilm assays

The ability of *E. coli* to form biofilms was assessed using a modified version of the microtitre dish biofilm formation assay originally described by O'Toole et al. (1999) and further detailed by O'Toole (2011). Strains were streaked out onto LB agar plates, with appropriate antibiotics for selection where required (Table 2.1) and incubated overnight at 37°C. A single colony was picked and used to inoculate 2 ml of LB (again supplemented with the appropriate antibiotic where necessary) which was then placed in a shaking incubator overnight at 37°C. Subsequently, the OD of the culture was measured at a wavelength of 600 nm, adjusted to an OD_{600} of 1, and diluted 100

fold in LB. A 200 µl aliquot of the normalised and diluted culture was added to the wells of polyvinyl chloride (PVC) microtitre plates (COSTAR®) equating to an inoculum of 2×10^6 CFU/well (200 µl). Plates were then placed in sterile airtight boxes and incubated at either 25°C or 37°C (in a static incubator) for 24 hours.

Biofilm formation was measured by CV staining. CV was used to stain biofilms as it binds to negatively charged ions; this means it stains bacterial cells as well as the ECM (including DNA and other negatively charged molecules) (Burmølle et al., 2006). After 24 hours incubation, all wells were washed once with 200 µl sterile water followed by staining with 200 µl 0.1% CV for 30 minutes. The CV solution was aspirated, with care taken so as not to disturb the biofilm adhered to the well, and the wells were then washed a further three times by the addition of 200 µl sterile water. Subsequently, 200 µl of 50% EtOH was added to each well and incubated for a further 30 minutes so as to solubilise the remaining CV stain. Finally, the depth of staining was measured using a Tecan Sunrise plate reader at a wavelength of 595 nm. The OD measurements taken were used as a proxy for the amount of biofilm present.

2.2.2 Competitive microtitre biofilm assay

In order to establish the presence of any population bottleneck in the microtitre plate biofilm assay, competitive biofilm assays were performed. These experiments were carried out using a modified version of the biofilm protocol previously described in Materials and Methods section 2.2.1. The inoculum for these assays was 2×10^6 CFU/ml constituted by varying proportions of WT UTI89 (SLC-6) and UTI89 containing a chromosomal kanamycin resistance marker (SLC-66). Briefly, each strain was grown up, from a single colony, in LB (supplemented with kan for SLC-66) over night at 37°C. Cultures were normalised to an OD_{600} of 1 and combined to make a final concentration of 2×10^6 CFU/200 µl in each well using the following proportions of SLC-66: 100%, 10%, 1%, 0.1%, 0.01%, 0.001%, 0.0001% and 0%. Three triplicate wells were prepared for each proportion. Biofilms were then allowed to form in the microtitre plates for 24 hours at 25°C and

37°C as previously described in Materials and Methods section 2.2.1. The bacterial load of each inoculum was confirmed by CFU enumeration (as previously described in Materials and Methods section 2.4.6) of a200 µl aliquot of the remaining inoculum, plating on both LB and LB/kan agar plates.

After 24 hours the planktonic cells were removed and the bacterial load for each well was determined by CFU enumeration (as described in Materials and Methods section 2.4.6) again plating on both LB agar and LB/kan plates. The empty wells were washed 10 times with 200 µl sterile 1X PBS (cooled to 4°C) and the attached biofilm cells were removed from the wells by scraping with sterile cotton swabs. A new swab was used for each well and after scraping, the swab heads were removed using sterile scissors. Each swab head was placed in 1 ml LB/kan and incubated at 37°C for 4 hours (in a shaking incubator). After 4 hours the bacterial load of each 1 ml swab culture was determined by CFU enumeration (as described in Materials and Methods section 2.4.6) plating on LB and LB/kan agar plates, as with the inocula and planktonic cells.

2.2.3 TraDIS biofilm assays

In order to determine the genes in UTI89 essential for biofilm formation at both 25˚C and 37˚C, transposon directed insertion site sequencing (TraDIS) was performed. As part of this procedure the UTI89 transposon mutant library (generated as described in Materials and Methods section 2.6.1) was passaged as follows:

A 50 ml (LB/kan) culture was started in a 250 ml Erlenmeyer flask from 2-3 inoculation loops of a frozen aliquot of the of the transposon mutant library. This was incubated overnight at 37°C in a shaking incubator. The TraDIS procedure was carried out in duplicate on different days each starting with a separate aliquot of the UTI89 transposon mutant library. The OD_{600} of the culture was measured and normalised to an OD_{600} of 1. Following this normalisation the cells were diluted 1/100. This cell suspension was aliquoted into 60 wells of a 96 well PVC microtitre plate (200 µl per

well), avoiding the outer most wells, resulting in a final cell concentration of 2×10^6 cells per well (CFU/200 µl). These outermost wells were filled with blank LB in order to monitor for any plate contamination.

The PVC plates were then placed in clean airtight boxes and incubated statically at either 25˚C or 37˚C for 24 hours. Following this incubation the planktonic cells were removed from all 60 wells of each plate and pooled (by plate). The wells were then washed 10 times with 200 µl sterile 1X PBS (cooled to 4°C) being careful to remove as much residual liquid as possible without disturbing the adhered biofilm cells. The biofilm was removed by scraping 30 wells with sterile cotton swabs. A total of 3 swabs were used per plate, with each swab being used for 10 wells, in order to prevent the swab heads from becoming too wet and to maximise the number of cells recovered. Swab heads were aseptically removed using sterile scissors and placed into a 2 ml ultracentrifuge tube.

DNA was extracted from the biofilm cells directly from the removed swab head and from the pooled planktonic cell suspension as described in Materials and Methods section 2.6.3.

2.3 Haemagglutination (HA) titres

The *fimH* adhesin on Type 1 pili is responsible for UPEC adherence to BECs (Krogfelt et al., 1990, Hung et al., 2002). Type 1 pili have also been shown to be able to bind to guinea pig red blood cells and cause haemagglutination; this haemagglutination is mannose sensitive (Duguid et al., 1955, Klemm, 1985). Haemagglutination titres were therefore carried out in order to test for the presence of functional type 1 pili and were performed as previously described by Hultgren et al. (1986) and Hultgren et al. (1990). A single colony was grown in 3 ml LB overnight at 37°C in a shaking incubator. Cultures were then normalised to an OD_{600} of 1. Subsequently, 1.5 ml of this standardised culture was centrifuged using a benchtop centrifuge (Eppendorf 5430) at 5000 rpm for 10 minutes and resuspended in 150 µl of cold PBS. A 25 µl aliquot of this cells suspension was added to the first

well of a V-bottomed microtitre plate (Corning®) along with 25 µl 1X PBS and then serially diluted 2 fold a further 11 times. Following this, 500 µl of guinea pig red blood cells (InVivos Pte Ltd.) were washed by suspension in 10 ml sterile 1X PBS followed by centrifugation in a benchtop centrifuge (Eppendorf 5804R) for 5 minutes at 5000 rpm. The supernatant was then discarded and the cells carefully resuspended in 1X PBS to a volume of 10 ml. Subsequently 25 µl of the red blood cells were added to each well and the plate was covered and kept at 4°C overnight to allow agglutination to occur. HA titre values were given as integers between 1 and 12 and were determined by counting across the wells until the last (least dilute well) to show any agglutination – the higher the value the more agglutination occurred.

2.4 Mouse Infections

2.4.1 Ethics statement

All animal experiments were performed by Shannon Fenlon in accordance with protocols that were reviewed and approved by the A*STAR Biological Resource Centre Institutional Animal Care and Use Committee (protocol #130853). These protocols were approved to be in accordance with the prevailing Singapore National Advisory Committee for Laboratory Animal Research (NACLAR) guidelines.

2.4.2 UTI model

Murine infections were carried out according to a previously published protocol (Hung et al., 2009) in order to determine the ability of UPEC strains and mutants to cause UTI in mice. Each strain was streaked out on to LB agar supplemented with the appropriate antibiotic (see Table 2.1) and incubated at 37°C overnight. Single colonies were selected and used to seed 10 ml cultures of LB (or BHI for *E. faecalis* – Table 2.1) in 100 ml Erlenmeyer flasks and incubated statically for 24 hours at 37°C. Cultures were subcultured 1:1000 into 5 ml LB (or BHI for *Enterococcus faecalis*) in a 50 ml Erlenmeyer flask and incubated statically for a further 24 hours at 37°C. The cultures were

subsequently centrifuged using a benchtop centrifuge (Eppendorf 5804R) and resuspended in 1X PBS to an OD_{600} of 0.5, resulting in bacterial suspension with a cell density of 2×10^7 CFU/50 µl to be used as the inoculum. A 50 µl aliquot of this bacterial suspension was used to transurethrally inoculate each 7-8 week old female C57BL/6J mouse as detailed below.

Inoculation was carried out using a ½ inch, 30G hypodermic needle, sheathed with polyethylene tubing, on a 1-ml tuberculin Slip-Tip syringe (needle, syringe and tubing all from BD Biosciences). The tip of the sheathed needle was lubricated with a sterile lubricating jelly, to minimise discomfort, and inserted transurethrally into the bladder of mice anesthetised with isoflurane. Finally, 50 µl of the prepared inoculum was slowly administered. A separate syringe and sheathed needle was utilised for each bacterial strain used.

Infected mice were sacrificed at 24 hours post infection (hpi) by isoflurane inhalation, followed by cervical dislocation. The mice were then laid on their backs and their abdomens sterilised with 100% EtOH prior to dissection to harvest the bladders and kidneys. The harvested bladders and kidneys were homogenized in 1 ml and 800 µl sterile 1X PBS respectively. Each homogenised sample was serially diluted and plated on LB agar (or BHI agar for *Enterococcus faecalis*) and the bacterial load was determined by CFU enumeration (See Materials and Methods section 2.4.6).

2.4.3 PCR to determine the phase orientation of *fimS*

The *fimS* element was amplified using the primers 5'-GAGAAGAAGCTTGATTTAACTAATTG-3' and 3'-AGAGCCGCTGTAGAACTCAGG-5'. The PCR reaction mixture was set up as follows: 41 µl water, 5 µl 10X ThermoPol buffer (New England BioLabs), 1 µl dNTP, 1 µl forward primer, 1 µl reverse primer, 0.5 µl *Taq* DNA polymerase and 1 µl of the inoculum cells. A Hinf1 restriction digest of this PCR product was then carried out. A 25 µl aliquot of the PCR reaction was taken, 1 µl of Hinf1 restriction enzyme (New England BioLabs) was added and the mixture incubated at 37°C for 1 hour.

A 5 µl aliquot of this reaction mixture was visualised by gel electrophoresis using a 1.5% agarose gel. Depending on the orientation of the *fimS* element (phase on or phase off) the digestion results in different sized fragments and a characteristic banding pattern is observed for both phase on and phase off cells.

2.4.4 IBC imaging

In IBC imaging experiments, mouse infections were carried out as described in Materials and Methods section 2.4.1, however bacteria expressing GFP (Table 2.1) were used for these infections. At the required time point infected mice were sacrificed as previously described, however in this case, bladders were harvested, carefully inverted, hemisected and finally splayed prior to viewing with an Olympus MVX10 fluorescence microscope.

2.4.5 CAUTI model

CAUTI mouse infections were carried out as described in Materials and Methods section 2.4 in the same way as the UTI model, but with the following alterations according to a previously published protocol (Guiton et al., 2010, Conover et al., 2015): Prior to bacterial inoculation mice were anaesthetised using isoflurane and their genital area sterilized with 100% EtOH. A 5 mm piece of silicone catheter was implanted into the bladder transurethrally by placing it on the end of a ½ inch, 30G hypodermic needle (attached to a tuberculin syringe) above a 7 mm piece of polyethylene tubing. The catheter implant was then lubricated and inserted into the mouse bladder transurethrally. Once in place the implant was pushed off the needle into the bladder, using forceps to move the polyethylene tubing in the direction of the bladder. Following this implantation, the inoculation was carried out as previously described in Materials and Methods section 2.4.2.

At 24 hpi the mice were sacrificed as previously described (section 2.4.2). Prior to dissection, the genital area was sterilised with 100% EtOH and a clean sheathed inoculation needle was inserted into the bladder transurethrally to push any catheter implants that may be sitting in the

urethra back into the bladder. Their abdomens were sterilised and bladders and kidneys harvested as previously described (section 2.4.1) however the implant was carefully, aseptically, recovered from the removed bladders and placed into 1 ml of sterile 1X PBS. Recovered catheters were then sonicated in 1 ml 1X PBS using a water bath sonicator (Bioruptor®) pulsing on high power for 15 minutes. The samples were subsequently vortexed for a further 10 minutes prior to serial dilution and CFU enumeration (see Materials and Methods section 2.4.6). Bladders and kidneys were homogenised as previously described in Materials and Methods section 2.4.2 and the bacterial load calculated according to Materials and Methods section 2.4.6.

2.4.6 CFU enumeration

A 200 µl aliquot of each 1 ml bacterial suspension (bladder and kidney homogenates and catheter sonicates) was added to the first row of a microtitre plate and 180 µl of cold sterile 1X PBS was added to all other wells in the column. Suspensions were serially diluted by 10X, by sequentially taking 20 µl from each well and adding it to the next well down the column, mixing well and changing pipette tips each time. Using a multichannel pipette 10 µl from each well in the column was spotted onto the appropriate agar plate (Table 2.1). This was repeated 5 times onto one agar plate, per sample, resulting in a total of 50 µl per well being plated for each sample. The plates were allowed to dry and incubated overnight at 37°C. CFUs were then counted from the lowest dilution to result in single countable colonies. The number of CFUs present in the 50 µl plated for this dilution was used to calculate the CFU/ml value for the original sample concentration. Using this method results in a limit of detection (LOD) of 20 CFU/ml as only 50 µl of the original 1 ml sample was directly measured.

2.5 Statistics

2.5.1 Crystal violet biofilm assays

The mean value for each group was calculated and plotted relative to the relevant WT strain Error bars on CV biofilm assay figures represent ± 1 SEM. Unpaired t-tests were performed, using GraphPad Prism 6 software, to determine the significance of differences in biofilm formation for each strain compared to the relevant WT strain at each temperature, *p*—values <0.05 were considered significant.

2.5.2 Growth Curves

The mean OD_{600} from three biological replicated was calculated and plotted for each strain at each time point. Unpaired t-tests were performed, using GraphPad Prism 6 software, to determine the significance of differences in absorbance (OD_{600}) between pairs of strains at each time point, *p*—values <0.05 were considered significant.

2.5.3 Animal experiments

The two-tailed Mann-Whitney U test was performed as described by Schwartz et al. (2015) and Blango and Mulvey (2010), using GraphPad Prism 6 software to compare median CFU values between groups of mice. The Mann-Whitney U test ranks all data points in ascending order and compares the average rank of each group in order determine significance. P values ≤0.05 were deemed to be significant. All values ≤LOD were given the LOD value of 20 (see Materials and Methods section 2.4.6) for the purpose of this test and graphical representation on a logarithmic scale. The Mann-Whitney U test (a non-parametric test) was used, so as not to assume a normal distribution.

2.6 Transposon directed insertion site sequencing (TraDIS)

In order to study the genes in UTI89 essential for biofilm formation, TraDIS was performed as described in the following subsections. The overall work flow for this TraDIS experiment is provided in Figure 2.2.

2.6.1 Generating a transposon mutant library in WT UTI89 (SLC-6)

A library of transposon mutants was created in UTI89 using a Tn5 transposome. The EZ-Tn5 <R6Kγ*ori*/KAN-2> transposome from Epicentre® (See Figure 2.1 for sequence) was transformed into WT UTI89 cells (SLC-6). In order to do this UTI89 cells were first made competent as previously described in Materials and Methods section 2.1.3. The competency of the cells was then tested by transformation of pUC57 (a derivative of pUC19) into an aliquot of the competent SLC-6 cells. In a 1 mm electroporation cuvette (BioRad), 6 ng pUC57 DNA was added to a 60 µl aliquot of the SLC-6 cells and electroporated using the Gene Pulser Xcell™ machine(BioRad) at the following settings: 1.7 kV, 25 µF, 400 Ω. Following electroporation, the cells were immediately resuspended in 1 ml pre-warmed LB (37°C) and incubated at 37°C for 2 hours in a shaking incubator. A 200 µl aliquot of the culture was then removed and titred to calculate the bacterial load as described in Materials and Method section 2.4.6. The cells were found to have a competency of 1.53×10^8 transformants per µg pUC57 and deemed to be sufficient for use in the generation of a UTI89 transposon mutant library.

Once the competency of the SLC-6 cells was deemed sufficient they were transformed with the EZ-Tn5 transposon by electroporation. Electroporation was carried out according to the transposome manufacturer's instructions. Briefly, 1 µl of transposome was added to a 60 µl aliquot of UTI89 competent cells and mixed by gentle pipetting up and down. This cell/transposome mixture was added to a 1 mm cuvette (BioRad) and electroporation was carried out using a Gene Pulser Xcell™ machine (BioRad) at the following settings: 1.7 kV, 25 µF, 400 Ω. Post electroporation

the cells were quickly resuspended in pre-warmed (37°C) SOC media (super optimal broth with glucose) (from Sigma Aldrich) and allowed to recover statically at 37°C for 2 hours before shaking at 37°C for a further 2 hours.

Following recovery, a 200 µl aliquot of the culture was taken and titred in order to determine the bacterial load (as previously described in Materials and Methods section 2.4.6). The rest of the culture was then spread across several large LB agar plates supplemented with 50 µg/µl kan and allowed to grow overnight. The titred bacteria were used to calculate the total number of transformants which was determined to be 3.2×10^5 CFU/ml. All colonies were then scraped from the agar plates into 10% glycerol, into five 1.5 ml aliquots and stored at -80˚C.

2.6.2 TraDIS biofilm screening assays

In TraDIS, in order to determine the genes essential for a given condition, transposon mutant libraries are screened in those conditions. In this thesis, TraDIS screening was carried out under a number of different conditions, namely; biofilm formation at 25°C and 37°C and growth in LB. These assays are detailed in the below sections.

2.6.2.1 TraDIS biofilm assays

TraDIS Biofilm assays were carried out as previously described in Materials and Methods section 2.2.3).

2.6.2.2 TraDIS growth in LB controls

TraDIS was also carried out for UTI89 growth in LB under static growth conditions as controls for the biofilm TraDIS experiment. A 50 ml (LB/kan) culture was started from 2-3 inoculation loops of a frozen aliquot of the transposon mutant library in a 250 ml Erlenmeyer flask. This was incubated overnight at 37°C in a shaking incubator (16 hours). The TraDIS procedure was carried out in duplicate on different days each starting with a separate aliquot of the UTI89 transposon mutant library. The OD_{600} of the culture was measured and normalised to an OD of 1

and subsequently diluted 100 fold. Two 200 µl aliquots of this cell suspension were used to start two individual 10 ml cultures (in LB) in two separate 100 ml Erlenmeyer flasks. The flasks were incubated statically, one at 25°C and one at 37°C for 24 hours.

2.6.3 TraDIS DNA extractions

All TraDIS DNA extractions were carried out using the Qiagen DNeasy extraction. Different protocols were used for biofilms and planktonic cells and those in liquid culture. Theses protocols are described in the following two sections, Materials and Methods 2.6.3.1 and 2.6.3.2.

2.6.3.1 Biofilm DNA extractions

Biofilm DNA was extracted using a slightly modified version of the DNA Mini Buccal Swab protocol from Qiagen as follows: The swab head was placed in a 2 ml microcentrifuge tube (as described in Materials and Methods section 2.6.2.1) and 400 µl of sterile 1X PBS was added, followed by 10 µl of RNase A/T1 mix (New England Biolabs) to ensure RNA free genomic DNA was extracted. Proteinase k (20 µl) was added followed by 400 µl of buffer AL (Qiagen). The mixture was incubated at 56°C for 10 minutes before 400 µl 100% EtOH was added and mixed by vortexing. A 700 µl aliquot of the swab mixture was added to a Qiagen spin column and centrifuged at 8000 rpm for 1 minute. The filtrate was discarded and the spin repeated with another 700 µl aliquot of the swab mixture in the same spin column and again the filtrate was discarded. Buffer AW1 (500 µl) was added the column and centrifuged for a further 1 minute at 8000 rpm and the flow through discarded. This was repeated with the addition of Buffer AW2 (500 µl) and centrifugation for 3 minutes at 14000 rpm. The column was centrifuged once more at full speed for 1 minute to ensure complete removal of all buffer. Finally the DNA was eluted from the spin column. The column was placed in a clean collection tube (1.5 ml ultracentrifuge tube) and 100 µl sterile nuclease free water was added to the filter and incubated at 42°C for 15 minutes and centrifuged for 1 minute at 8000 rpm. This was then repeated with a further 100 µl nuclease free water, resulting in a final volume of 200 µl eluate for each swab head and 600 µl for each biofilm test plate.

DNA eluates for each biofilm test plate were then pooled and the DNA concentrated using Amicon® Ultra Centrifugal Filters according to the manufacturer's protocol.

2.6.3.2 Planktonic and liquid culture extractions

DNA extractions from all pooled planktonic cells and liquid culture (including the mutant library after 16 hours of growth in LB that was used to seed TraDIS screening experiments) were carried out using the QIAamp DNA Mini Kit (Qiagen) according to the 'Tissue Protocol' provided by the manufacture with a few alterations due to the larger volume of these samples. Each sample was transferred to falcon tubes and pelleted in a cooled benchtop centrifuge (Eppendorf 5804R) for 5 minutes at 10000 rpm. The supernatants were removed and the pellets resuspended in 3 ml – 5 ml of buffer ATL (lysis buffer from Qiagen), depending on the size of the pellet. The appropriate amount of proteinase k was added to the samples (based on their volume) and the samples incubated at 56°C for at least 3 hours, with regular vortexing until fully lysed. The appropriate volume of RNase was added (again depending on the sample volume) and samples were incubated at 70°C for 10 minutes. EtOH was then added in the correct proportion depending on sample volume and the samples were transferred in 500 µl aliquots into separate Qiagen spin filters. The Qiamp DNA Mini Tissue Protocol was then followed as published and samples were eluted in 200 µl sterile water following a 15 minute incubation at 42°C. The multiple eluates for each sample were then combined and concentrated by passing through Amicon® Ultra Centrifugal Filters (following the manufacturer's protocol).

2.6.4 TraDIS library preparation and sequencing

Sequencing library preparation for TraDIS was carried out as described in the below sections using Illumina Truseq nanoLT kits according to a modified version of the manufacturer's protocol, based on the methodology used by Phan et al. (2013).

2.6.4.1 DNA Fragmentation

The concentration of each DNA sample was ascertained using the Qubit system from Invitrogen. Qubit is a DNA quantification system that uses a fluorescent DNA intercalating dye to directly measure the amount of DNA present in a sample and is widely considered to be much more accurate than methods that measure A260/280 ratios in order to quantify DNA. The DNA was then diluted to 100 ng in a final volume of 52.5 µl Illumina resuspension buffer. The DNA was then sheared using a Covaris Ultrasonicator (LE220) to an average size of 350 bp at the following settings – duty factor: 15%, peak incident power: 450 W, cycles per burst: 200, treatment time: 130 seconds.

The sheared DNA was transferred into 1.5 ml ultracentrifuge tubes and cleaned up by the addition of 80 µl Sample Purification Beads and mixed by careful pipetting up and down, followed by a 5 minute incubation at room temperature. The beads were separated from the mixture by placing the tubes on a magnetic rack (DynaMag™-2) for 8 minutes. When the samples were clear the supernatant was carefully removed and the beads washed twice with 200 µl 80% EtOH, incubating the beads for 30 seconds at room temperature in the EtOH each time. Any residual EtOH was then removed with a 10 µl pipette and the beads were allowed to air-dry for 5-10 minutes.

Once dry the 62.5 µl Resuspension buffer was added to the beads, the tubes were removed from the magnetic rack and the beads resuspended in the buffer by gentle pipetting up and down. The mixture was incubated for 2 minutes at room temperature before being placed back on the magnetic rack for 5 minutes until the samples were clear. A 60 µl volume of each sample was then transferred to a clean 1.5 ml ultracentrifuge tube.

2.6.4.2 End repair and size selection

End Repair was carried out in order to blunt the sticky ends left over from the mechanical DNA shearing protocol. End Repair Mix 2 (40 µl) was added to the fragmented and cleaned up DNA and mixed by careful pipetting up and down and mixture incubated for 30 minutes at 30°C using a thermocycler. Size selection was performed as follows: Sample Purification beads (2622 µl) were

diluted with nuclease free water (1794 µl) according the Illumina protocol. A 160 µl aliquot of the diluted bead mixture was added to the end repair mixture and incubated at room temperature for 5 minutes. The mixture was placed on the magnetic stand and allowed to clear. Once clear 125 µl of the supernatant was removed and placed in a clean 1.5 ml microcentrifuge tube. This was repeated with a further 125 µl of sample resulting in a new tube containing 250 µl of the DNA of interest.

Small DNA fragments were removed by the addition of 30 µl undiluted Sample Purification Beads and incubated for 5 minutes at room temperature. The mixture was placed on the magnetic stand, and once clear, the supernatant was carefully removed and discarded, and the beads washed twice with 80% EtOH before being allowed to dry. They were then resuspended in 20 µl Illumina resuspension buffer and incubated at room temperature for 2 minutes before being placed back on the magnetic stand and allowed to clear. Once clear, 17.5 µl of the supernatant from each sample was recovered and placed in clean PCR tubes.

2.6.4.3 Adenylation of 3' ends

To prevent self-ligation of the DNA fragments during adapter ligation (section 2.6.4.3) the 3' end of the blunt fragments were adenylated. A-Tailing Mix (12.5 µl) was added to DNA and mixed by pipetting. The tubes were placed in a thermocycler and incubated at 37°C for 30 minutes, the temperature raised to 70°C for a further 5 minutes followed by 4°C for 5 minutes and finally carried straight through to adapter ligation.

2.6.4.4 Adapter ligation

Resuspension buffer (2.5 µl) was added to the adenylated DNA samples along with 2.5 µl Ligation Mix 2 and 2.5 µl Adapter (one unique Illumina adapter per sample) and mixed by pipetting. Tubes were then incubated at 30°C for 10 minutes. The reaction was stopped by the addition of 5 µl stop ligation buffer. The DNA was then cleaned up from the reaction mixture. Sample Purification Beads (42.5 µl) were added to the reaction mixture, mixed thoroughly and incubated for 5 minutes

at room temperature. The mixture was placed on the magnetic rack and allowed to clear and the supernatant removed. The remaining bead pellet was washed twice with 80% EtOH. The beads were then allowed to dry and resuspended in 52.5 µl resuspension buffer before being placed back on the magnetic rack. The cleared supernatant (50 µl) was transferred to a clean tube and a second clean up step was completed using 50 µl of sample collection beads before elution in a final volume of 25 µl Resuspension Buffer.

2.6.4.5 Transposon specific library enrichment

The enrichment step from the Illumina protocol was replaced with a transposon specific enrichment step as described by Phan et al. (2013). This transposon specific amplification was carried out using the KAPA library amplification kit and a transposon specific primer. This transposon specific primer was used as the forward primer and designed to amplify the end of the transposon sequence out into the genomic DNA, resulting in a library of DNA fragments primed for sequencing representing the transposon insertion junctions. In order to achieve this, transposon specific primers contained: the Illumina P5 sequence to allow the DNA fragment to bind to the sequencing flow cell; Illumina sequencing primer 1 to allow sequencing to take place; a 6bp custom index (these have the same sequences as Illumina's sequencing indexes); and finally a transposon specific sequence. The location of the transposon specific part of the primer within the transposon sequence is displayed in Figure 2.1. The reverse primer used was the llumina Index 1 reverse primer. All TraDIS primers are detailed in Table 2.3. The reaction was set up as follow : 25 µl 2X KAPA HiFi hot start mix, 2.5 µl Illumina index primer 1 (Table 2.3), 2.5 µl transposon specific primer (Table 2.3) and 20 µl DNA after ligation. The PCR reaction carried out using the following thermocycling conditions: one initial denaturation cycle at 98°C for 45 seconds followed by 22 cycles of denaturation at 98°C for 15 seconds, annealing at 65°C for 30 seconds and extension at 72°C for 30 seconds. One final extension was carried out at 72°C for 1 minute.

```
1     CTGTCTCTTA  TACACATCTC  AACCATCATC  GATGAATTGC  TTCGTTAATA
51    CAGATGTAGG  TGTTCCACAG  GGTAGCCAGC  AGCATCCTGC  GATGCAGATC
101   CGGATGCCAT  TTCATTACCT  CTTTCTCCGC  ACCCGACATA  GATCCGAAGA
151   TCAGCAGTTC  AACCTGTTGA  TAGTACGTAC  TAAGCTCTCA  TGTTTCACGT
201   ACTAAGCTCT  CATGTTTAAC  GTACTAAGCT  CTCATGTTTA  ACGAACTAAA
251   CCCTCATGGC  TAACGTACTA  AGCTCTCATG  GCTAACGTAC  TAAGCTCTCA
301   TGTTTCACGT  ACTAAGCTCT  CATGTTTGAA  CAATAAAATT  AATATAAATC
351   AGCAACTTAA  ATAGCCTCTA  AGGTTTTAAG  TTTTATAAGA  AAAAAAAGAA
401   TATATAAGGC  TTTTAAAGCT  TTTAAGGTTT  AACGGTTGTG  GACAACAAGC
451   CAGGGATCTG  CCATTTCATT  ACCTCTTTCT  CCGCACCCGA  CATAGATCCG
501   GAACATAATG  GTGCAGGGCG  CTGACTTCCG  CGTTTCCAGA  CTTTACGAAA
551   CACGGAAACC  GAAGACCATT  CATGTTGTTG  CTCAGGTCGC  AGACGTTTTG
601   CAGCAGCAGT  CGCTTCACGT  TCGCTCGCGT  ATCGGTGATT  CATTCTGCTA
651   ACCAGTAAGG  CAACCCCGCC  AGCCTAGCCG  GGTCCTCAAC  GACAGGAGCA
701   CGATCATGCG  CACCCGTGGC  CAGGACCCAA  CGCTGCCCGA  GATGCGCCGC
751   GTGCGGCTGC  TGGAGATGGC  GGACGCGATG  GATATGTTCT  GCCAAGGGTT
801   GGTTTGCGCA  TTCACAGGGT  GTCTCAAAAT  CTCTGATGTT  ACATTGCACA
851   AGATAAAAAT  ATATCATCAT  GAACAATAAA  ACTGTCTGCT  TACATAAACA
901   GTAATACAAG  GGGTGTTATG  AGCCATATTC  AACGGGAAAC  GTCTTGCTCG
951   AGGCCGCGAT  TAAATTCCAA  CATGGATGCT  GATTTATATG  GGTATAAATG
1001  GGCTCGCGAT  AATGTCGGGC  AATCAGGTGC  GACAATCTAT  CGATTGTATG
1051  GGAAGCCCGA  TGCGCCAGAG  TTGTTTCTGA  AACATGGCAA  AGGTAGCGTT
1101  GCCAATGATG  TTACAGATGA  GATGGTCAGA  CTAAACTGGC  TGACGGAATT
1151  TATGCCTCTT  CCGACCATCA  AGCATTTTAT  CCGTACTCCT  GATGATGCAT
1201  GGTTACTCAC  CACTGCGATC  CCCGGAAAAA  CAGCATTCCA  GGTATTAGAA
1251  GAATATCCTG  ATTCAGGTGA  AAATATTGTT  GATGCGCTGG  CAGTGTTCCT
1301  GCGCCGGTTG  CATTCGATTC  CTGTTTGTAA  TTGTCCTTTT  AACAGCGATC
1351  GCGTATTTCG  TCTCGCTCAG  GCGCAATCAC  GAATGAATAA  CGGTTTGGTT
1401  GATGCGAGTG  ATTTTGATGA  CGAGCGTAAT  GGCTGGCCTG  TTGAACAAGT
1451  CTGGAAAGAA  ATGCATAAAC  TTTTGCCATT  CTCACCGGAT  TCAGTCGTCA
1501  CTCATGGTGA  TTTCTCACTT  GATAACCTTA  TTTTTGACGA  GGGGAAATTA
1551  ATAGGTTGTA  TTGATGTTGG  ACGAGTCGGA  ATCGCAGACC  GATACCAGGA
1601  TCTTGCCATC  CTATGGAACT  GCCTCGGTGA  GTTTTCTCCT  TCATTACAGA
1651  AACGGCTTTT  TCAAAAATAT  GGTATTGATA  ATCCTGATAT  GAATAAATTG
1701  CAGTTTCATT  TGATGCTCGA  TGAGTTTTTC  TAATCAGAAT  TGGTTAATTG
1751  GTTGTAACAC  TGGCAGAGCA  TTACGCTGAC  TTGACGGGAC  GGCGGCTTTG
1801  TTGAATAAAT  CGAACTTTTG  CTGAGTTGAA  GGATCAGATC  ACGCATCTTC
1851  CCGACAACGC  AGACCGTTCC  GTGGCAAAGC  AAAAGTTCAA  AATCACCAAC
1901  TGGTCCACCT  ACAACAAAGC  TCTCATCAAC  CGTGGCGGGG  ATCCTCTAGA
1951  GTCGACCTGC  AGGCATGCAA  GCTTCAGGGT  TGAGATGTGT  ATAAGAGACA
2001  G
```

Figure 2.1 EZ-Tn5™ <R6Kγori /KAN-2> Sequence

The EZ-Tn5™ <R6Kγori /KAN-2> was used to generate a UTI89 mutant library in this thesis. The sequence of this transposon is provided overleaf here. Bases highlighted in yellow are those in the transposon specific primer used in the TraDIS sequencing library preparation (Table 2.3) and the first round of demultiplexing (DEMUX 1). Bases underlined in Red are those that were used for the second round of demultiplexing (DEMUX 2). Full demultiplexing methodology and subsequent analysis are detailed in Materials and Methods section 0.

Table 2.3 Primers used for TraDIS library transposon specific amplification.

Primer name	Direction	Illumina P5 / Illumina sequencing primer 1 / Illumina Index / Tn5 specific sequence (excluding reverse primer)
TraDIS_Tn5_AD001	Forward	AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT ATCACG ACCTACAACAAAGCTCTCATCAACC
TraDIS_Tn5_AD002	Forward	AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT CGATGT ACCTACAACAAAGCTCTCATCAACC
TraDIS_Tn5_AD003	Forward	AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT TTAGGC ACCTACAACAAAGCTCTCATCAACC
TraDIS_Tn5_AD004	Forward	AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT TGACCA ACCTACAACAAAGCTCTCATCAACC
TraDIS_Tn5_AD005	Forward	AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT ACAGTG ACCTACAACAAAGCTCTCATCAACC
TraDIS_Tn5_AD006	Forward	AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT GCCAAT ACCTACAACAAAGCTCTCATCAACC
TraDIS_Tn5_AD007	Forward	AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT CAGATC ACCTACAACAAAGCTCTCATCAACC
TraDIS_Tn5_AD008	Forward	AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT ACTTGA ACCTACAACAAAGCTCTCATCAACC
TraDIS_Tn5_AD009	Forward	AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT GATCAG ACCTACAACAAAGCTCTCATCAACC
TraDIS_Tn5_AD010	Forward	AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT TAGCTT ACCTACAACAAAGCTCTCATCAACC
TraDIS_Tn5_AD011	Forward	AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT GGCTAC ACCTACAACAAAGCTCTCATCAACC
TraDIS_Tn5_AD012	Forward	AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT CTTGTA ACCTACAACAAAGCTCTCATCAACC
Primer name	**Direction**	**Illumina P5 / Illumina sequencing primer 1 / Illumina Index / Tn5 specific sequence (excluding reverse primer)**

TraDIS_Tn5_AD013	Forward	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT AGTCAA ACCTACAACAAAGCTCTCATCAACC
TraDIS_Tn5_AD014	Forward	AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT AGTTCC ACCTACAACAAAGCTCTCATCAACC
TraDIS_Tn5_AD015	Forward	AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT ATGTCA ACCTACAACAAAGCTCTCATCAACC
TraDIS_Tn5_AD016	Forward	AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT CCGTCC ACCTACAACAAAGCTCTCATCAACC
TraDIS_Tn5_AD018	Forward	AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT GTCCGC ACCTACAACAAAGCTCTCATCAACC
TraDIS_Tn5_AD019	Forward	AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT GTGAAA ACCTACAACAAAGCTCTCATCAACC
TraDIS_Tn5_AD020	Forward	AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT GTGGCC ACCTACAACAAAGCTCTCATCAACC
TraDIS_Tn5_AD021	Forward	AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT GTTTCG ACCTACAACAAAGCTCTCATCAACC
TraDIS_Tn5_AD022	Forward	AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT CGTACG ACCTACAACAAAGCTCTCATCAACC
TraDIS_Tn5_AD023	Forward	AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT GAGTGG ACCTACAACAAAGCTCTCATCAACC
TraDIS_Tn5_AD025	Forward	AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT ACTGAT ACCTACAACAAAGCTCTCATCAACC
TraDIS_Tn5_AD027	Forward	AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT ATTCCT ACCTACAACAAAGCTCTCATCAACC
Illumina Index 1 primer	Reverse	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTC

Following this transposon specific PCR amplification, DNA was recovered from the reaction mixture with Sample Purification Beads and cleaned up according to the Illumina Truseq DNA Nano LT kit protocol as follows: Sample Purification Beads (50 µl) were added to the PCR reaction mixture, mixed thoroughly and incubated at room temperature for 5 minutes. Samples were placed on the magnetic rack and allowed to clear. Once clear 95 µl of the supernatant was discarded and the beads washed twice with 80% EtOH. All residual ethanol was removed and the beads allowed to dry. When dry, the beads were resuspended in 32.5 µl resuspension buffer, then mixed thoroughly and incubated at room temperature for a further 2 minutes. The samples were placed back on the magnetic stand and after 5 minutes 30 µl of the supernatant (constituting the final sequencing library) was transferred to a clean tube. All Illumina sequencing libraries were stored at -20˚C until library QC, normalisation and pooling (described in Materials and Methods section 2.6.4.6).

2.6.4.6 Sequencing library QC, normalisation and pooling

Once constructed, sequencing libraries were subject to a quality control (QC) procedure in order to ensure libraries were of sufficient quality and quantity for Illumina sequencing. QC was carried out by electrophoresis on the Agilent 2100 Bioanalyzer system and using DNA 1000 Agilent kits. QC was performed according to the Agilent DNA 1000 protocol using 1 µl of each prepared TraDIS sequencing library. The Agilent profile for each of the samples was used to check that the sequencing libraries contained the correct size fragments as detailed in the Illumina Truseq Nano Library Prep Guide. The DNA concentration obtained by Agilent electrophoresis was then used to normalise each of the samples to a concentration of 10 nM. The normalised samples were pooled for sequencing by mixing 5 µl of each into one tube. This pooled TraDIS library was then sent for next generation sequencing (NGS) by the Genome Institute of Singapore (GIS) NGS sequencing platform.

2.6.4.7 TraDIS Sequencing

Two biological replicates of the TraDIS screening were carried out using two separate aliquots of the original mutant library. All samples, including replicates, were then sequenced on one sequencing run. Paired end sequencing was performed by the Genome Institute of Singapore NGS sequencing platform (core facility) using the Hiseq 4000 instrument on one lane with a read length of 2X151 bp.

2.6.5 TraDIS data analysis

TraDIS sequencing data was initially demultiplexed by Swaine Chen according to the custom index present and the transposon specific primer sequence at the 5' end of the read (31 bp total) introduced during the transposon specific library amplification step as described in section 2.6.4.5 and depicted in Figure 2.1 and. This was achieved by trimming the 31bp index and transposon primer sequence and using it as an index read to demultiplex using deML (Renaud et al., 2015). The data were then subject to a second round of de-multiplexing using the 69 bp remainder of the transposon specific sequence present in the reads and again the 69 bp trimmed and used as an index read for demultiplexing with deML (the transposon sequence used for this de-multiplexing is indicated in Figure 2.1).

De-multiplexed libraries were then aligned to the UTI89 reference genome sequence (NC_007946) and plasmid sequence (NC_007941) (Chen et al., 2006) using Burrows-Wheeler Aligner (BWA) version 0.1.10 (Li and Durbin, 2009) using the BWA-MEM algorithm with default parameters. The BAM files were then filtered by SAM Flags to obtain the first in pair of properly mapped paired reads (reads with flag 64 but not flag 4). The filtered bam files were then used to determine the insertion positions, and for the reads that mapped to the reverse strand the insertion position was corrected by the length of the read. The number of reads at each position was then collated in a table. The number of reads per gene was determined by aggregating the data by position using the ddply function in R (plyr package version 1.8.4). A proportion test was carried to

compare the number of reads for each gene between libraries in order to determine the likelihood that they differ. This was performed using the prop.test function in the base R package using the two sided option and otherwise default parameters (version 3.0.0).

Subsequent analysis was performed by Shannon Fenlon: Summary statistics of the BAM files resulting from sequence alignment were determined using the SAMtools version 1.1 (Li et al., 2009) flagstat function. The *p* values obtained by the proportion test were used to generate Q-Q plots in R (Base package version 3.0.0) using the following function described by Turner (2010) for each pairwise comparison:

```
ggd.qqplot =
function(pvector,
main=NULL, ...) {
                    o = -log10(sort(pvector,decreasing=F))
                    e = -log10( 1:length(o)/length(o) )
                    plot(e,o,pch=19,cex=1, main=main, ...,
                        xlab=expression(Expected~~-log[10](italic(p))),
                        ylab=expression(Observed~~-log[10](italic(p))),
                        xlim=c(0,max(e)), ylim=c(0,max(o)))
                    lines(e,e,col="red")
                }
```

The *p* values were then corrected in R using the Benjamini-Hochberg approach (Benjamini and Hochberg, 1995). This was performed using the p.adjust function and the BH method with default parameters. The most significant differences (corrected *p* values) in read number for each comparison were then determined using the order function R (Base package, version 3.0.0).

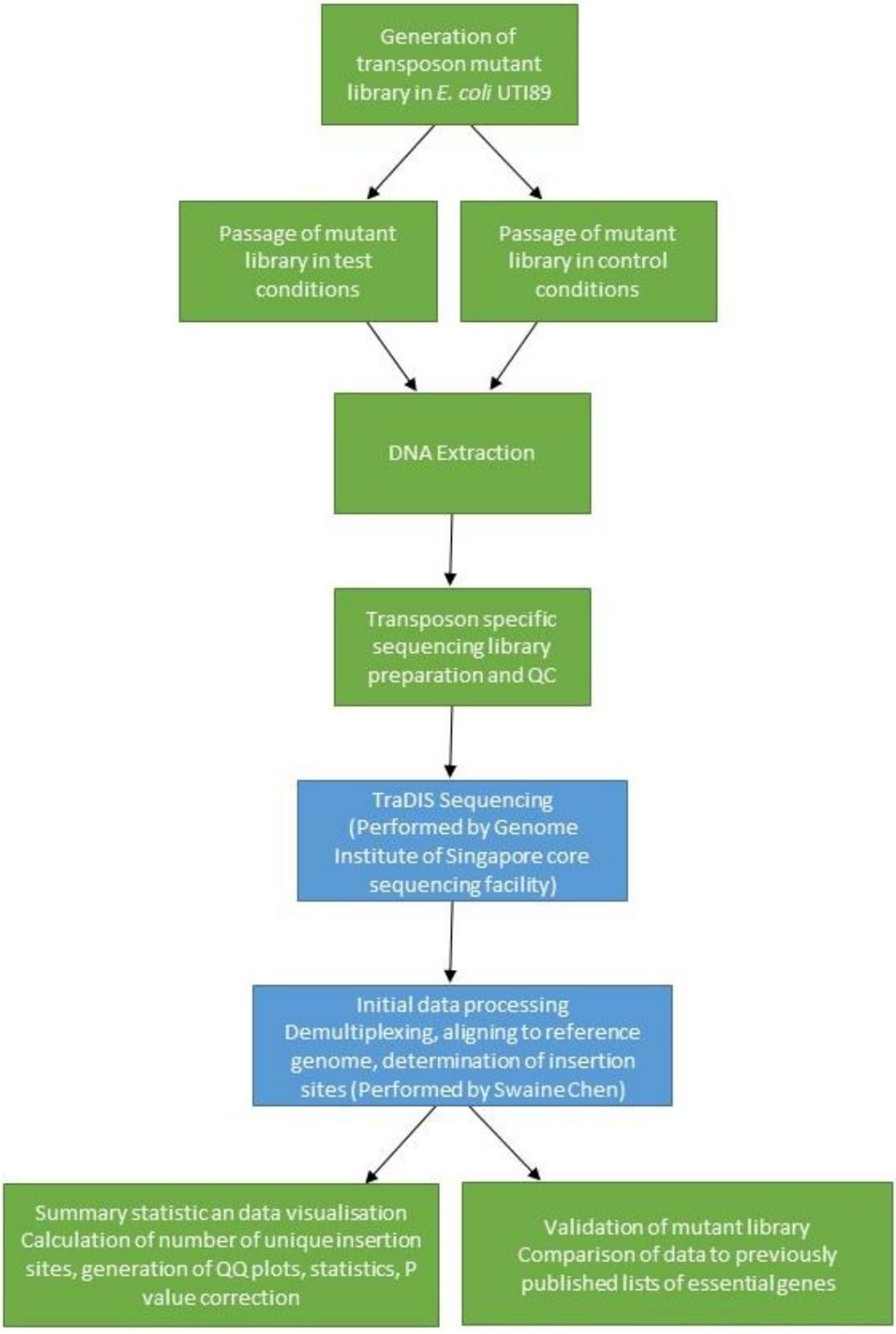


Figure 2.2 TraDIS experimental workflow

Flowchart describing the overall workflow for TraDIS experiments. Green boxes indicate work performed by Shannon Fenlon, and blue boxes indicate work performed by others and state who it was that performed this work.

Chapter 3: Genetic Characterisation of a Temperature Dependent Biofilm

3.1 Introduction

Type 1 pili are reported to be a crucial virulence factor for UPEC, and are pivotal in UPEC pathogenesis. As previously described in Introduction section 1.4.1.1, they are essential for UPEC attachment to BECs and their subsequent invasion and IBC formation (Connell et al., 1996, Martinez et al., 2000, Schwartz et al., 2011, Anderson et al., 2003). Biofilm assays are commonly used to model type 1 pili function *in vitro;* in a microtitre biofilm assay, biofilm formation has been shown to be dependent on type 1 pili (Pratt and Kolter, 1998). However, an initial observation in the Chen lab (Majid Eshaghi & Swaine Chen, personal communication, September 2013) highlighted a possible difference in the biofilm formation abilities of UTI89 at varying temperatures that had not been previously described in literature, whereby a type 1 pili deficient strain was able to form biofilms at 37°C. The incubation temperature used for microtitre biofilm assays varies between previously published studies (Table 1.2). This variation makes it difficult to compare published studies, and caution should be taken when interpreting data from the various assays. The majority of published CV biofilm assays assessing type 1 pili are carried out at 25°C, despite 37°C being the more physiologically relevant temperature for human infection.

The potential, uncharacterised, difference in biofilm formation at 37°C supports the idea that in CAUTI, biofilm formation on abiotic surfaces (catheters) may not be entirely dependent on type 1 pili (unlike IBC formation in UTIs) and suggests that the microtitre plate biofilm assay at 25°C may not be the most appropriate *in vitro* model for biofilm formation relating to CAUTI. The pathogenesis of UTI has been very well characterised and is well understood. Type 1 pili have been shown as crucial in this context and as a result, it has previously been suggested that type 1 pili may also play a pivotal role in CAUTI (Mobley et al., 1987). The exact pathogenesis of CAUTI however is

less well studied in comparison (Jacobsen et al., 2008) and the ability of type 1 pili deficient UPEC to form biofilms at 37°C contradicts this idea suggesting that there may be another important mechanism for biofilm formation relevant to human infections, such as CAUTI. A better understanding of the biofilm formation abilities of UPEC strains and the models used for their study is crucial for the further study and understanding of CAUTI pathogenesis.

3.2 Aims

The major aims of this chapter are to validate and characterise an initial observation that biofilm formation varies with temperature in an *in vitro* model of biofilm formation (Majid Eshaghi & Swaine Chen, personal communication, September 2013) and to ascertain the importance of type 1 pili for biofilm formation at both 25°C and 37°C. This will be achieved using different attachment surfaces, genetic mutants and chemical inhibition studies.

3.3 Hypotheses

1. The dependence of UPEC strain UTI89 on type 1 pili for biofilm formation varies with temperature
2. Biofilm formation is type 1 pili independent in an *in vitro* CV microtitre plate biofilm assay at 37°C.
3. The varying dependence on type 1 pili for biofilm formation with temperature is not affected by the adherence material.

3.4 Results

3.4.1 UTI89 formed type 1 pili independent biofilms at 37°C

An initial observation was made that *in vitro* biofilm formation appeared to be type 1 pili independent at 37°C (Majid Eshaghi & Swaine Chen, personal communication, September 2013). This was unexpected as previously published data had previously shown biofilm formation to be type 1 pili dependent at 25°C in the same *in vitro* assay (Lo et al., 2014, Hadjifrangiskou et al., 2012, Pratt and Kolter, 1998, Schembri et al., 2001).

In order to examine the mechanism behind this observation in a more controlled manner, the biofilm formation capabilities of the clinical cystitis strain UTI89 and several type 1 pili mutants were assessed at both 25°C and 37°C using the CV microtitre plate assay as detailed in Materials and Methods section 2.2.1. WT UTI89 forms biofilms and the biofilm formation ability of UTI89Δ*fim* (deletion of the full *fim* operon encoding type 1 pili), was significantly reduced (p = <0.0001) relative to WT UTI89. This supported previously published data (Lo et al., 2014, Hadjifrangiskou et al., 2012, Pratt and Kolter, 1998) and suggests biofilm formation is dependent on type 1 pili at 25°C. In order to confirm that this phenotype was due to the disruption of type 1 pili several other mutants were tested as controls and a significant reduction in biofilm formation was observed at 25°C. These mutants included two strains, Q133K (p = <0.0001) and A62S (p = <0.0001), with single point mutations in the FimH adhesin affecting their binding capacity, and a phase locked off strain (p = <0.0001) where the type 1 pili coding sequencing was intact but its expression was locked off (with the *fimS* element in the reverse orientation) due to mutations in the IR sequences that prevent recombination and therefore phase variation (see Introduction section 1.4.1.1).

The ability of the same strains (WT UTI89, UTI89Δ*fim,* Q133K, A62S, and phase off) to form biofilms were also tested at 37°C in the same assay (Figure 3.1b). WT UTI89 was able to form biofilms at 37°C. However, at this temperature biofilm formation of deletion of the full *fim* operon

strain (UTI89Δ*fim*), single FimH point mutation A62S and the phase locked off strain did not differ significantly to that of wildtype (p = 0.0883, p = 0.7182 and p = 0.1164 respectively) whilst the biofilm formation of UTI89 Q133K was 120% that of WT UTI89 (p = 0.0084) (Figure 3.1b) suggesting that biofilm formation at 37°C was type 1 pili independent.

Published CV biofilm assays that have been carried out at 25°C are often incubated for a longer time period than the 24 hours most often used for those incubated at 37°C (Table 1.2). In order to confirm that the type 1 pili independent phenotype observed at 37°C was not an artefact resulting from the length of incubation, the experiment was also carried out at both 25°C and 37°C with an incubation time of 48 hours (Figure 3.1c and d respectively). The same phenotypes, reduction of biofilm formation relative to WT UTI89 type 1 pili defective strains at 25°C and no reduction in biofilm formation relative to WT UTI89 at 37°C, were observed at 48 hours, as at 24 hours, with most of the strains tested. The only exception was the Q133K mutant incubated at 37°C for 48 hours which formed significantly less biofilm that WT UTI89 (p = 0.0003) however this observation was not repeated (data not shown). As the same suspected type 1 pili independent biofilm formation phenotype at 37°C was observed at 24 and 48 hours, 24 hours was used for all subsequent CV assays described in this thesis.

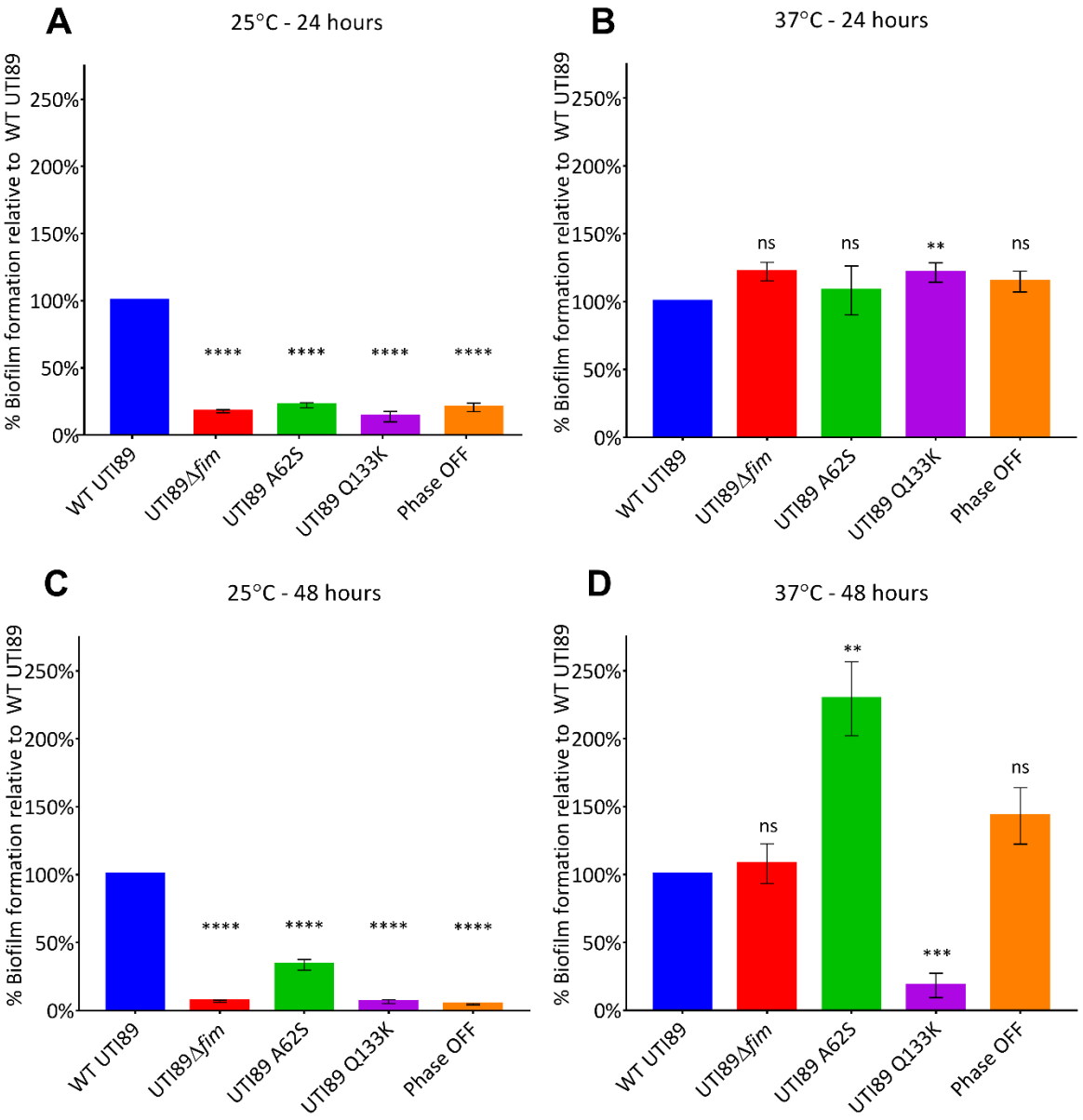


Figure 3.1 Biofilm formation of WT UTI89 and several type 1 pili mutants at 25°C and 37°C after 24 hours and 48 hours incubation.

Bar graphs showing *in vitro* biofilm formation, on PVC plates, of various UTI89 type 1 pili mutants (detailed in Table 2.1) relative to the wild type (WT UTI89) after 24 hour incubation at 25°C (a) or 37°C (b) as well as after 48 hours at 25°C (c) or 37°C (d). Biofilm formation was measured by CV staining of bacteria attached to PVC mictrotitre plates after incubation at 25°C and 37°C for 24 and 48 hours. The OD_{600} reading for CV staining of each strain was normalised to that of WT UTI89. Each bar represents at least 6 technical replicates with the error bars representing ± 1 SEM. An unpaired t-test was performed to compare the biofilm formation of each stain to UTI89 WT at each condition. *p* values ≤0.05 were considered significant. Asterisks denote level of significance * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

At 25°C the attenuated biofilm formation phenotype of UTI89Δ*fim* was partially rescued by complementation on a plasmid (Figure 3.2). The fim complement strain was generated by Majid Eshaghi by transformation of UTI89Δfim with a plasmid constitutively expressing the full type 1 pili operon and exhibits 60% biofilm formation relative to WT UTI89 ($p = <0.0001$) whilst biofilm formation of UTI89Δfim was less than 10% that of WT UTI89 ($p = <0.0001$). The reduced biofilm phenotype of the Q133K FimH mutant ($p = <0.0001$) was also able to be rescued at 25°C (achieved by reversion at the native locus with no residual markers) with this strain showing a slight increase (13%) in biofilm formation compared to WT UTI89 ($p = 0.0035$), confirming that this single point mutation does reduce biofilm formation at 25°C. As previously shown in Figure 3.1, locking the phase variable *fimS* element into the off position again prevented biofilm formation at 25°C ($p = <0.0001$) (Figure 3.2), however a phase locked on strain, generated in the same way, is able to form biofilms at 114% that of WT UTI89 ($p = 0.0012$). In contrast, at 37˚C all of the type 1 pili deficient strains used (UTI89 Δ*fim*, Q133K and phase off) are able to form biofilms at the same level as, or significantly more than, WT UTI89 (Figure 3.2).

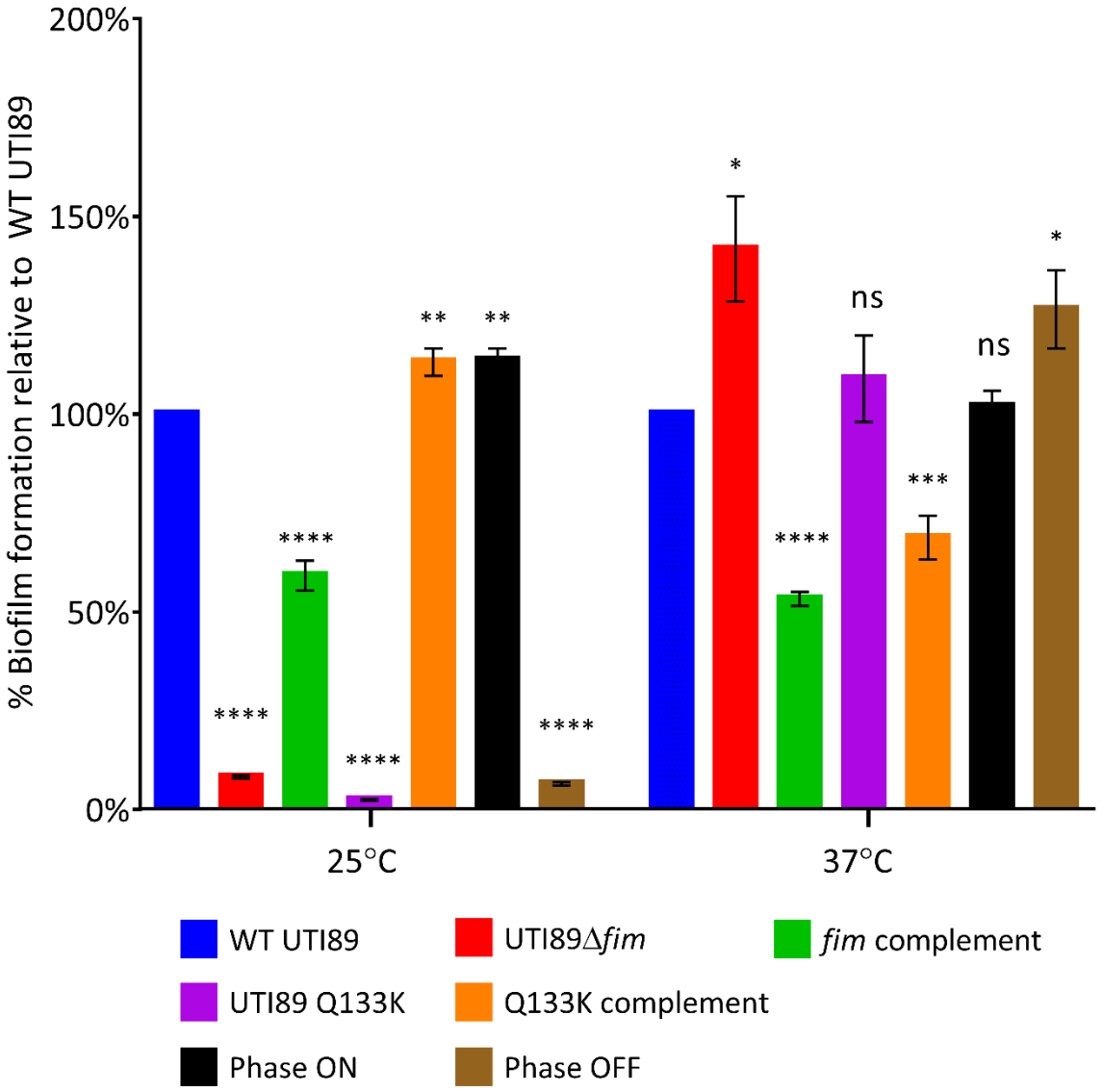


Figure 3.2 Complementation rescued attenuated biofilm phenotype of type 1 pili mutants at 25°C

Bar graphs showing *in vitro* biofilm formation, on PVC plates, of various UTI89 type 1 pili mutants and their respective complement strains (detailed in Table 2.1) after a 24 hour incubation at either 25°C or 37°C Biofilm levels are displayed relative to WT UTI89 biofilm formation at the respective temperature.Biofilm formation was measured by CV staining of bacteria attached to PVC mictrotitre plates after incubation at 25°C and 37°C for 24 hours. The OD_{600} reading for CV staining of each strain was normalised to that of WT UTI89. Each bar represents at least 6 technical replicates with the error bars representing ± 1 SEM. An unpaired t-test was performed to compare the biofilm formation of each stain to UTI89 WT at each condition. *p* values ≤0.05 were considered significant. Asterisks denote level of significance * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Genetic manipulation indicated that biofilm formation of UTI89 is type 1 pili dependent at 25°C but independent of type 1 pili at 37°C (Figure 3.1 and Figure 3.2). In order to confirm these observations a chemical inhibition test was performed and biofilm assays were carried out, as previously described, with the addition of mannose to the media. Mannose binding ability is often used to confirm the presence of type 1 pili. This is because the FimH adhesin at the tip of the type 1 pilus binds mannose (Hultgren et al., 1986, Krogfelt et al., 1990) – an ability shown to allow the binding of UTI89 to BECs (Krogfelt et al., 1990). The addition of mannose has been shown to inhibit type 1 pili mediated adhesion to abiotic surfaces *in vitro* by competitive binding of the FimH adhesin (Blumer et al., 2005, Pratt and Kolter, 1998).

At 25°C the addition of 4% mannose to the growth media significantly reduced biofilm formation by WT UTI89 compared to un-supplemented LB (p = 0.0004) further indicating that this biofilm is type 1 pili dependent. WT UT89 biofilm formation is not prevented by the addition of mannose at 37°C, in fact it is increased under these conditions compared to standard LB (p= 0.0095) but did little to reduce type 1 pili independent biofilm (Figure 3.3). The raised biofilm formation seen in the mutant strains at 25°C was most likely due to the usage of the additional sugar as a carbon source resulting in increased growth.

As a control, the same chemical inhibition experiment was carried out using glucose in the place of mannose (at the same concentration) in order to confirm that the effect of mannose was due to interaction with the FimH adhesin and not as a result of an extra sugar source in the growth media, the FimH adhesin is mannose specific and so does not bind glucose. Biofilm formation of WT UTI89 was not reduced in the presence of glucose as it was in the presence of mannose. Increased biofilm formation in the presence of glucose has previously been described by Pratt and Kolter (1998).

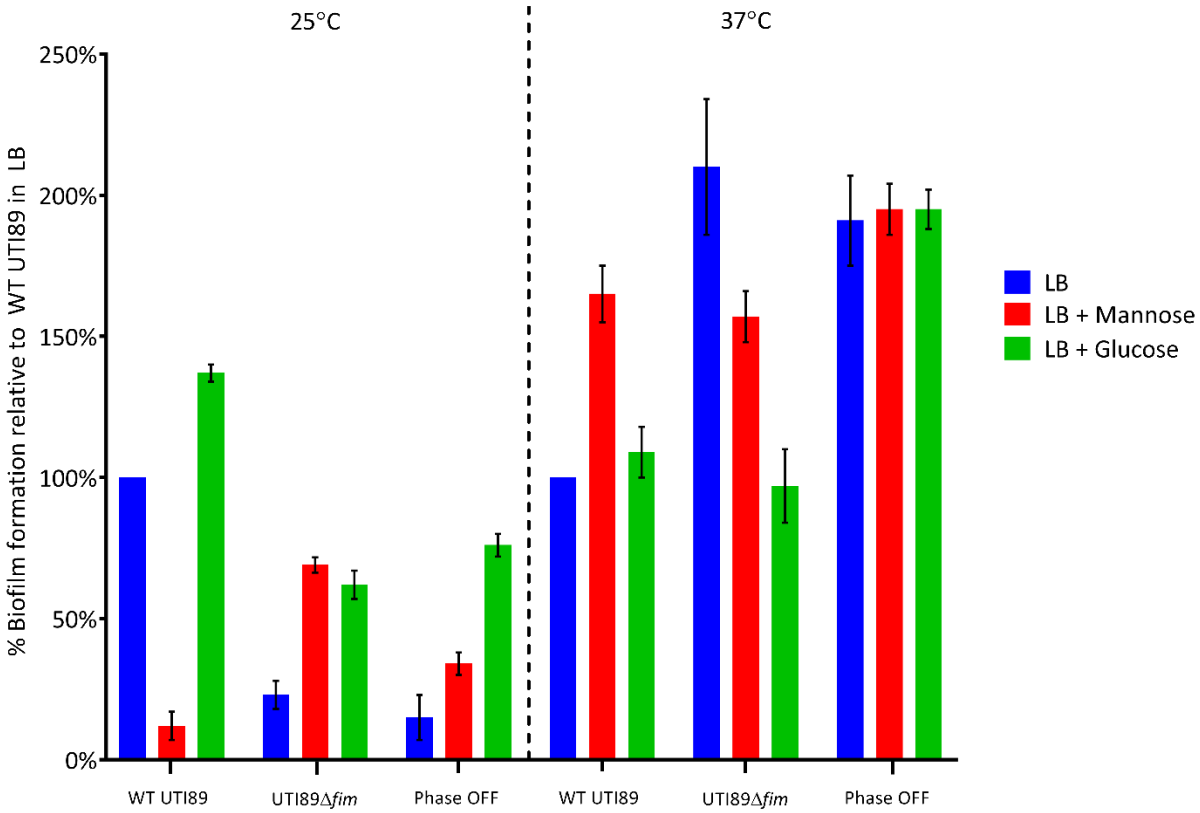


Figure 3.3 Mannose inhibits biofilm formation at 25°C but doesn't have an inhibitory effect on biofilm formation at 37°C.

The addition of 4% mannose to media of CV biofilm assays reduces type 1 pili dependent biofilm formation at 25°C (a) but not the type 1 pili independent biofilm formation seen at 37°C (b). Biofilm formation levels shown here are the OD_{600} readings of CV staining of cells adhered to PVC plates after 24 hours incubation, displayed relative to SLC-6 (wild type UTI89) in standard LB media at each temperature. Each bar represents at least 3 technical replicates and error bars represent ± 1 SEM.

3.4.2 Type 1 pili independent biofilm formation occurred specifically at 37°C.

In catheterised patients, the *in situ* catheter temperature ranges from room temperature (around 25°C) to 37°C, the latter of which relates to being inside the GU tract. The sections in between are likely to be at an intermediate temperature as they are outside of the body but in contact with it externally. Having established a difference in dependence on type 1 pili for biofilm formation between those formed *in vitro* at 25°C and 37°C, biofilm assays were repeated at temperatures between 20°C and 42°C (as described in Materials and Methods section 2.2.1) in order to test the requirement for type one pili for biofilm formation at these temperatures. A UTI89 Q133K mutant, UTI89Δ*fim* and a phase locked off UTI89 strain displayed significantly reduced biofilm formation at 20°C (p = <0.0001 for all three strains compared to UTI89 WT at 20°C), 25°C (p = <0.0001 for all three strains compared to UTI89 WT 25°C) and 30°C (p = <0.0001 for all three strains compared to UTI89 WT 30°C). At 42°C UTI89 Q133K and UTI89Δ*fim* formed significantly less biofilm compare to WT UTI89 at this temperature (p = <0.0001 for both), however the difference was less pronounced than at 20°C, 25°C and 30°C. The phase locked off strain on the other hand showed a slight decrease in biofilm formation compare to WT UTI89 but this was not statistically significant. These attenuated biofilm phenotypes for each strain were successfully rescued by complementation or reversion, suggesting that type 1 pili are required for biofilm formation at all 4 of these temperatures. In contrast, at 37°C none of the mutants tested displayed attenuated biofilm formation (Figure 3.4). UTI89Δ*fim, fim* complement, phase locked on and phase locked off displayed an increase in biofilm formation relative to WT UTI89 at 37°C (p = 0.0056, p = <0.0001, p = <0.0001 and p = <0.0001 respectively), whilst Q133K and its complement strain displayed no significant difference in biofilm formation compared to WT UTI89 at this temperature further suggesting that type 1 pili are not required for biofilm formation at 37°C. In summary, of the temperatures tested, it was only at 37°C that biofilm formation was not attenuated in the absence of functional type 1 pili.

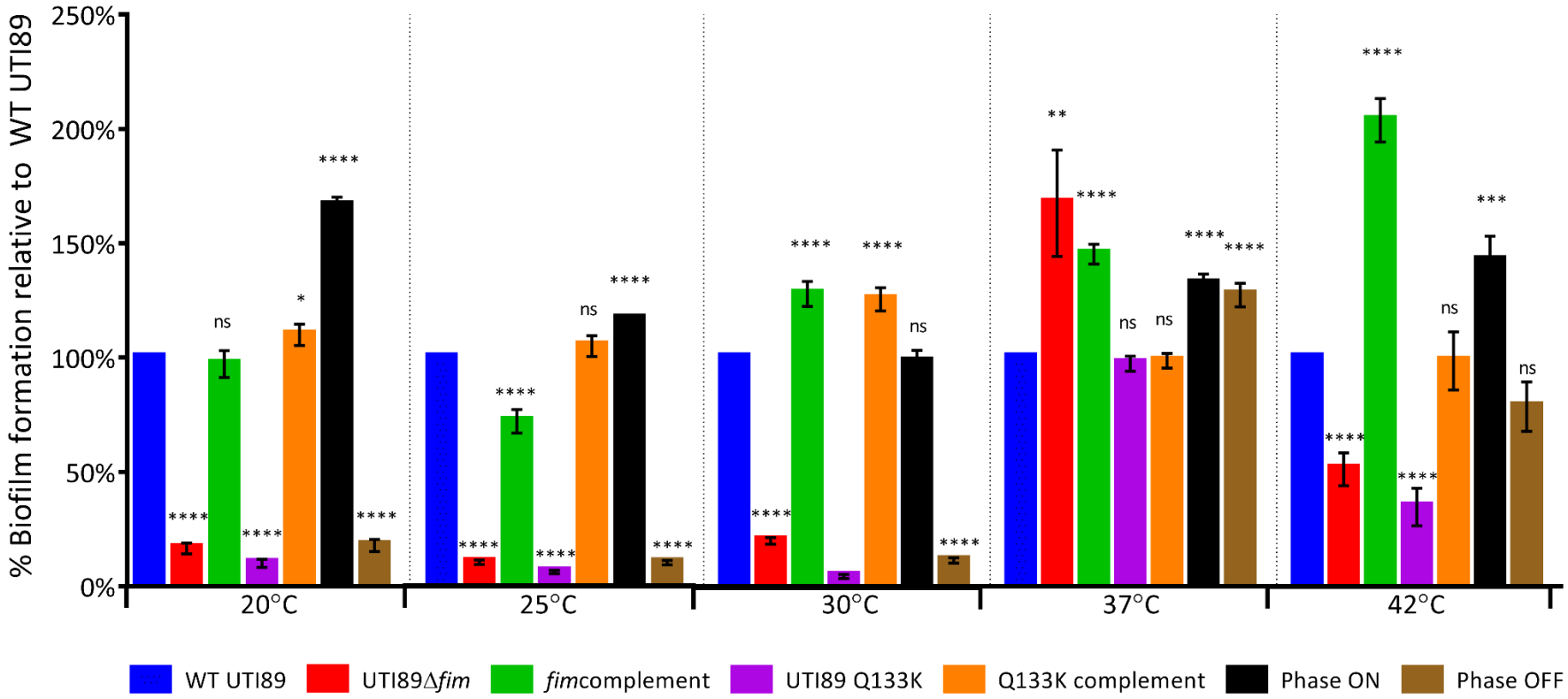


Figure 3.4 Type 1 pili dependent biofilm formation was specific to 37˚C.

Bar graph showing the biofilm formation of WT UTI89 and various type 1 pili mutant strains at various temperature intervals between 20°C and 42°C. Biofilm formation displayed is the OD_{600} of CV staining of cells adhered to PVC plates after 24 hour incubation, relative to that of WT UTI89 at each temperature. Each bar represents 6 technical replicates (individual wells) from 3 biological replicates and the error bars represent ± 1 SEM. An unpaired t-test was performed to compare the biofilm formation of each stain to UTI89 WT at each condition. *p* values ≤0.05 were considered significant. Asterisks denote level of significance * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

3.4.3 Biofilm formation at 37°C was independent of curli, flagella and S pili as well as type 1 pili.

Other than type 1 pili, many other factors have been implicated in the biofilm formation of UPEC strains, including curli, flagella and other CUP pili such as S pili (see Introduction section 1.4.1.1). The biofilm formation abilities of curli, flagella and S pilus genetic mutants were tested in order to establish whether or not these factors play a role in the type 1 pili independent biofilm formation observed at 37°C. The biofilm formation of curli mutants was assessed at both 25°C and 37°C *in vitro.*

As previously described (Introduction section 1.4.1.1.4) curli have been shown to be important for UPEC biofilm formation as they constitute a considerable proportion of the ECM (McCrate et al., 2013). As the expression of curli has been shown to vary with temperature (Szabo et al., 2005), they were considered to be a candidate for the type 1 pili independent biofilm formation observed at 37°C. Curli mutant strains were generated by deletion of the entire c*sgDEFG* operon, which has been shown to prevent curli synthesis and expression (Hammar et al., 1995). Deletion of the full c*sgDEFG* operon (UTI89Δ*csg*) did not attenuate biofilm formation at 37°C. Biofilm formation in this strain was 139% that of WT UTI89 at the same temperature (p = 0.0012), (Figure 3.5a). A double knockout of both the *csgDEFG* and *fim* operons (UTI89Δ*fim*Δ*csg*) also did not prevent or attenuate biofilm formation at 37°C with biofilm formation in this strain at 237% that of WT UTI89 (p=0.0040) (Figure 3.5b), whilst biofilm formation of the same strain was 5% of WT UTI89 (p=<0.0001).

Flagella are another factor commonly implicated in UPEC pathogenesis and biofilm formation (Introduction section 1.4.1.1.1) (Schwan, 2008, Lane et al., 2007, Wright et al., 2007, Pratt and Kolter, 1998). The *fliC* gene encodes the main structural sub-unit of the flagellum and deletion of this gene prevents the synthesis of functional flagella and renders cells immotile. The ability of a flagella deficient UTI89 strain (UTI89Δ*fliC*) to form biofilms in the CV biofilm assay was tested and

found not to attenuate the increased biofilm formation observed at 37°C and biofilm formation was 119% that of WT UTI89 at this temperature (*p=0.0137* (Figure 3.6)).

Like type 1 pili, S pili are classified as CUP pili owing to the process by which they are assembled and expressed on the cell surface. There is evidence to suggest that S pili expression is inverse to that of type 1 pili with S pilus expression having been shown to be increased when type 1 pili are not present (Greene et al., 2014, Kostakioti et al., 2009). This inverse relationship in expression between type 1 pili and S pili cast S pili as a potential candidate for type 1 pili independent biofilm formation at 37°C. As a result, a UTI89 mutant strain deficient in S pili was used to test biofilm formation at both 25°C and 37°C. Deletion of the entire S pilus operon (*sfaCBADEFGSH*) resulted in a slight reduction in biofilm formation at both 25°C and 37°C (Figure 3.7) relative to WT UTI89 at the respective temperature (94%, p = 0.0425 and 77%, p= 0.0075 respectively).

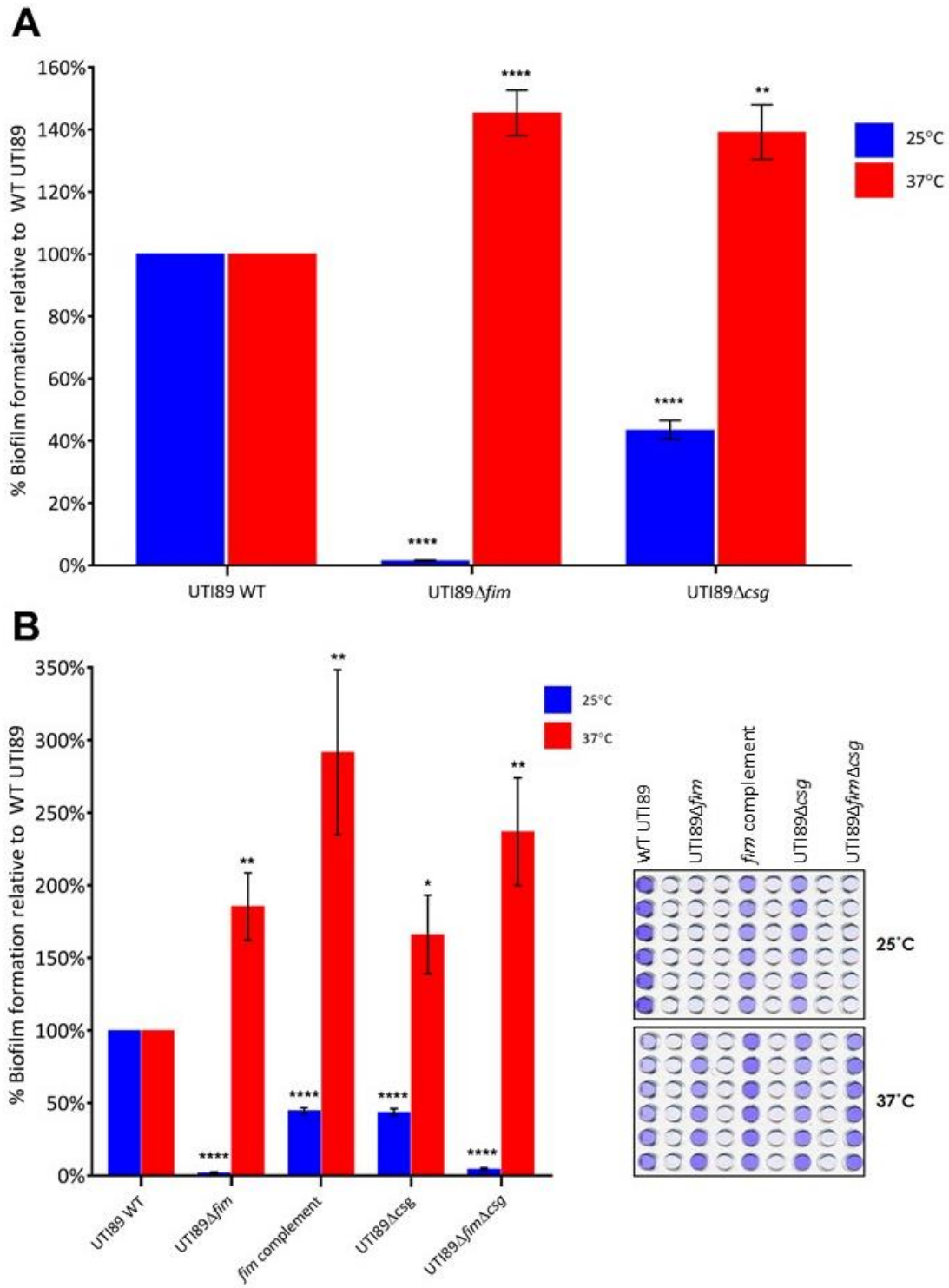


Figure 3.5 Biofilm formation at 37˚C was curli independent.

Bar graphs in (a) and (b) show OD_{600} of crystal violet staining of cells adhered to PVC after 24 hour incubation at either 25˚C or 37˚C, displayed relative to WT UTI89 at each temperature. Each bar represents at least 6 technical replicates and the error bars represent ± 1 SEM. An unpaired t-test was performed to compare the biofilm formation of each stain to UTI89 WT at each condition. *p* values ≤0.05 were considered significant. Asterisks denote level of significance * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Image in panel (b) is a photograph of the CV staining in the microtitre plates, matching the data in the bar graph shown in the same panel. Both the *csg* mutant (a) and a double *fim* and *csg* operon knock out (b) were able to form biofilms at 37˚C on PVC plates, indicating that biofilm formation at 37˚C was independent of type 1 pili.

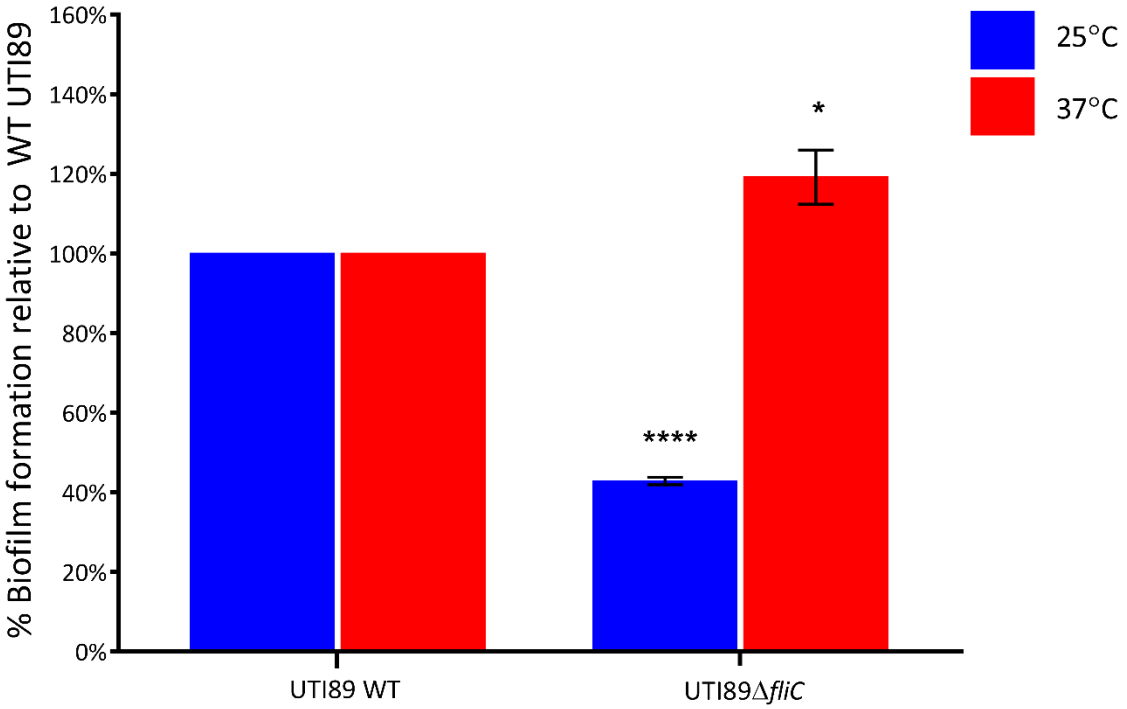


Figure 3.6 Biofilm formation at 37˚C was flagella independent

Bar graph showing the ability of a *fliC* mutant to form biofilms at 37˚C and 25˚C on PVC plates. Biofilm formation levels are shown as the mean OD_{600} of crystal violet staining of cells adhered to PVC plates after 24 hour incubation, relative to WT UTI89 at each temperature. Each bar represents at least 6 technical replicates and the error bars represent ± 1 SEM. An unpaired t-test was performed to compare the biofilm formation of each stain to UTI89 WT at each condition. *p* values ≤0.05 were considered significant. Asterisks denote level of significance * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

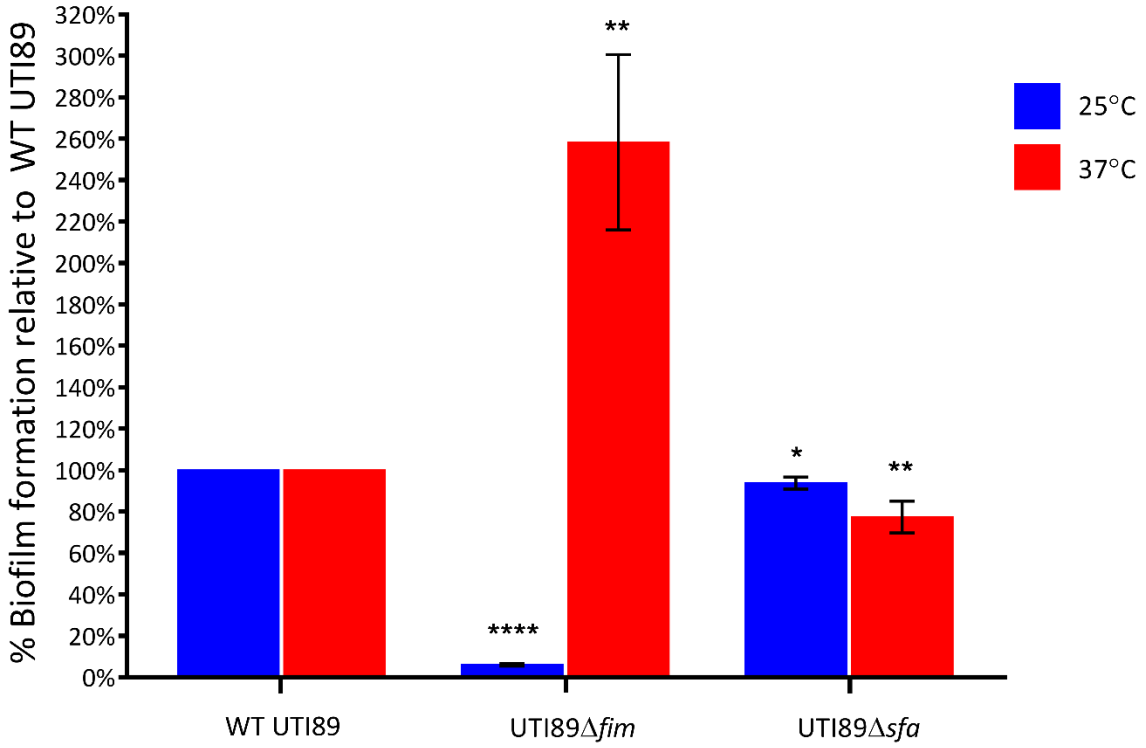


Figure 3.7 Mutation of S pilus does not substantially reduce biofilm formation at either 25°C or 37°C.

Bar graph showing the ability of UTI89Δ*sfa* (a full S pilus operon knockout) to for biofilms at 25˚C and 37˚C, on PVC plates, relative to wildtype UTI89 at each temperature. Biofilm levels are shown as the mean OD_{600} of crystal violet staining of cells adhered to PVC plates after 24 hours of incubation relative to WT UTI89 at either 25°C or 37°C. Each bar represents at least 6 technical replicates from two independent experiments and the error bars represent ± 1 SEM. An unpaired t-test was performed to compare the biofilm formation of each stain to UTI89 WT at each condition. *p* values ≤0.05 were considered significant. Asterisks denote level of significance, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

3.4.4 Type 1 pili independent biofilm formation at 37˚C was observed in CFT073 but not in K12 (MG1655)

The importance of type 1 pili and other *E. coli* virulence factors are often studied in other *E. coli* strains. As such the biofilm formation abilities of two other *E. coli* strains CFT073 and MG1655 (K-12) and their respective type 1 pili mutants were tested *in vitro* at both 25°C and 37°C. CFT073, like UTI89, is a clinical UPEC isolate; unlike UTI89 however, CFT073 was isolated from the blood of a patient with a suspected upper urinary tract infection (pyelonephritis) (Mobley et al., 1990). In contrast to UTI89 and CFT073, MG1655, a K-12 derivative, is a laboratory maintained prototypic *E. coli* strain. Like UTI89, CFT073 and MG1655 have previously been shown to express functional type 1 pili (Gally et al., 1993, Gunther et al., 2001). Biofilm formation by CFT073Δ*fimH* is significantly reduced at 25°C relative to WT CFT073 (p= <0.0001) whereas at 37°C the same strain shows no significant difference in biofilm formation relative to WT CFT073 (p= 0.8843). This suggests that whilst they are required for CT073 *in vitro* biofilm formation at 25°C, type 1 pili are not required for biofilm formation *in vitro* at 37°C. In contrast, deletion of the *fim* operon in the laboratory strain MG1655 (*MG1655Δfim*) significantly reduces biofilm formation at both 25˚C and 37˚C to 3% (p= <0.0001) and 8% (p= <0.0001) that of WT respectively (Figure 3.9) suggesting that type 1 pili are required at both temperatures for biofilm formation by this strain.

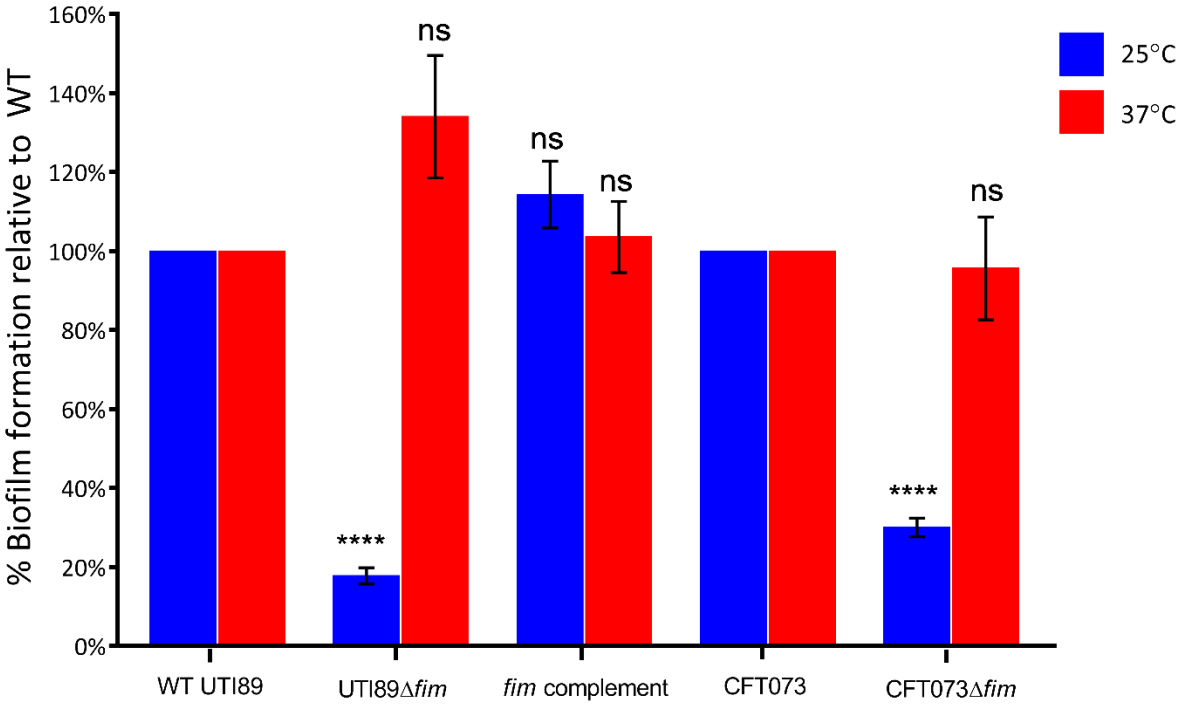


Figure 3.8 CFT073 form biofilms in the absence of functional type 1 pili at 37°C.

Bar graph showing the biofilm formation of UTI89 and CFT073 and their respective *fim* mutants on PVC plates after growth at 25˚C and 37˚C. Biofilm formation levels shown are the mean OD_{600} values of CV staining of cells adhered to PVC plates after 24 hours incubation, relative to the WT of each strain at each temperature. Each bar represents at least 6 technical replicates from three individual experiments with the error bars represent ± 1 SEM. An unpaired t-test was performed to compare the biofilm formation of each stain to UTI89 WT at each condition. *p* values ≤0.05 were considered significant. Asterisks denote level of significance * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

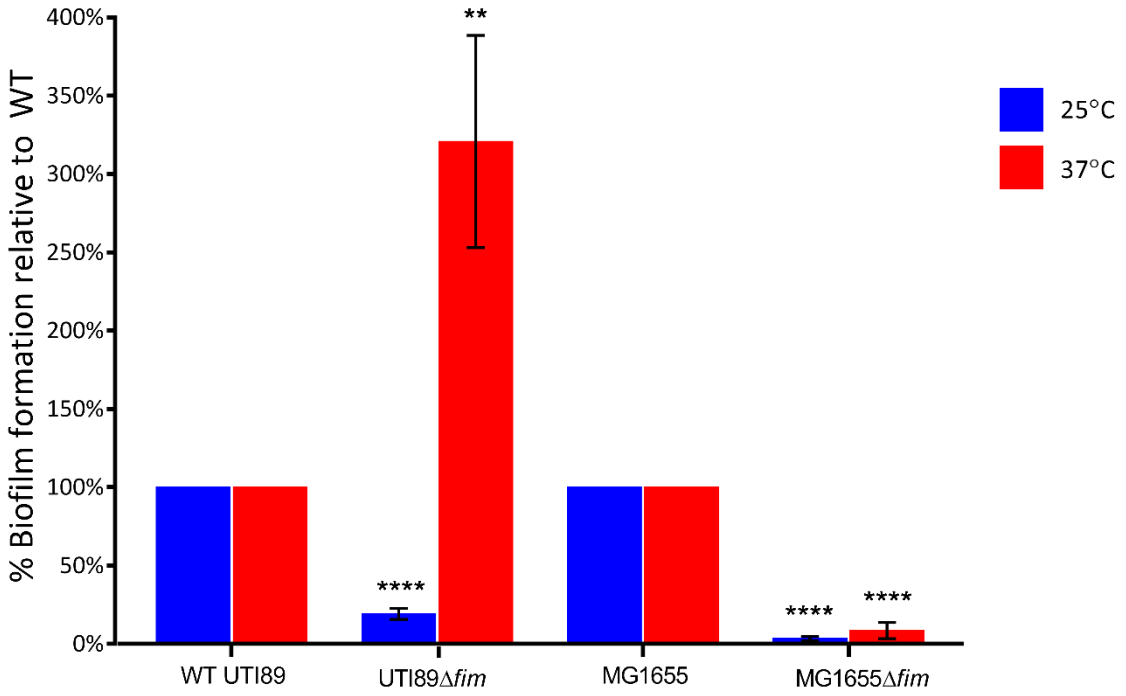


Figure 3.9 MG1655 is unable to form biofilms in the absence of type 1 pili at either 25°C or 37°C

Bar graph showing the biofilm formation of UTI89 and MG1655 and their respective *fim* mutants on PVC plates. An MG1655 strain with a type 1 pili mutation (SLC-153) was unable to form biofilms at either 25°C or 37°C relative to its WT strain (SLC-7).

Biofilm formation levels shown are the mean OD_{600} values of CV staining of cells adhered to PVC plates after 24 hours incubation, relative to the WT of each strain at each temperature. Each bar represents between three and six technical replicates from four individual experiments with the error bars representing ± 1 SEM. An unpaired t-test was performed to compare the biofilm formation of each stain to UTI89 WT at each condition. p values ≤0.05 were considered significant. Asterisks denote level of significance * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

3.4.5 The adherence material used for biofilm assays did not affect the observed phenotype

Catheters used in the clinical setting can be made from a number of materials, the most common of these is silicone, although PVC, and silicone coated PVC, amongst others, may also be used depending on the circumstances of each individual patient (International Continence Society., 2013). So far in this thesis, the adherence material used for *in vitro* biofilm characterisation using the CV assay has been PVC. In order to investigate whether or not the phenotype observed (type 1 pili independent biofilm formation at 37°C) is a general phenotype or specific to the adherence material used, the CV assay was repeated (as previously described in Materials and Methods section 2.2.1) using microtitre plates of a different material. The ideal material for this experiment would be silicone. However, silicone microtitre plates are not commercially available in the same format as other plates used in this project and CV staining was not possible on silicone catheter sections. Polystyrene plates were therefore used instead to assess the type 1 pili independent biofilm phenotype on another abiotic surface other than the PVC already used.

On polystyrene plates at 25°C UTI89Δ*fim* formed 0% biofilm compared to WT UTI89 (p= <0.0001), whereas at 37°C the same strain performed much better, forming 372% biofilm relative to WT UTI89 (p = 0.0088). This indicates that in the absence of type 1 pili, biofilms form at 37°C on polystyrene (as with PVC plates as previously shown) and raises the possibility that type 1 pili independent biofilm formation may occur on a range of different abiotic surfaces and is not necessarily specific to PVC as an adherence material.

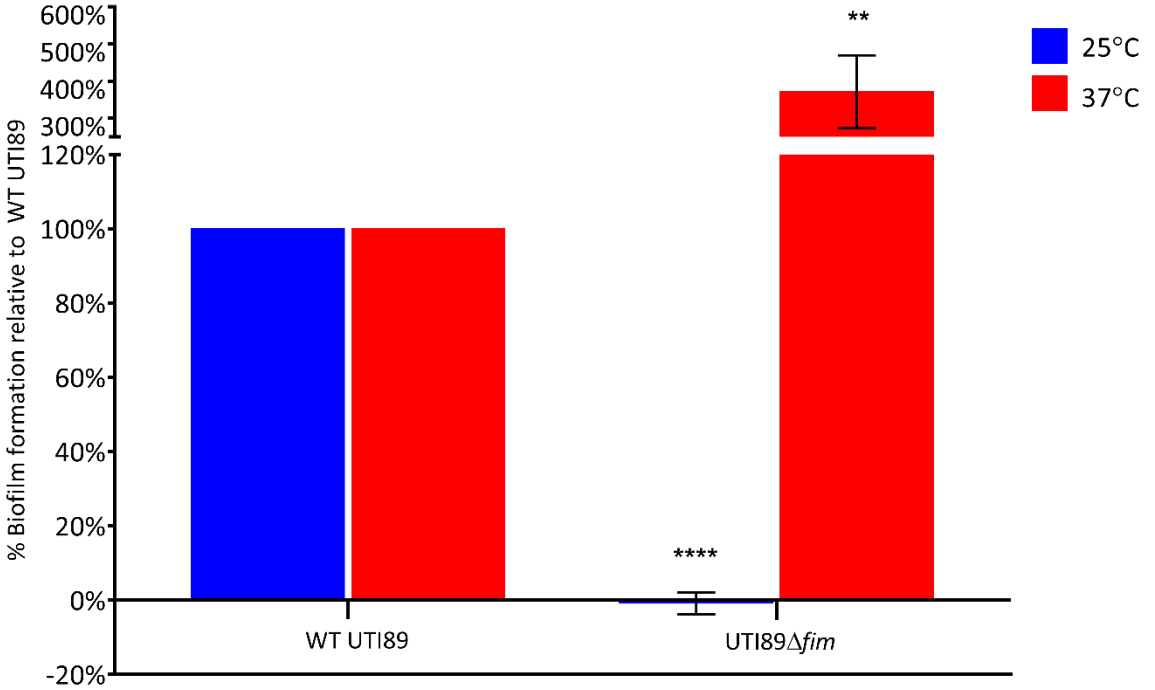


Figure 3.10 Type 1 pili independent biofilm formation was also observed at 37˚C on polystyrene plates.

Bar graph to show the biofilm formation of WT UTI89 and UTI89Δ*fim* at both 25˚C and 37˚C on polystyrene plates Biofilm formation is shown here as the mean OD_{600} of CV staining of cells adhered to polystyrene plates after 24 hours incubation, relative to WT UTI89 at both temperatures. Each bar represents at least 6 technical replicates from multiple experiments and the error bars represent ± 1 SEM. An unpaired t-test was performed to compare the biofilm formation of each stain to UTI89 WT at each condition. *p* values ≤0.05 were considered significant. Asterisks denote level of significance * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

3.4.6 Functional type 1 pili were expressed at 37°C prior to biofilm formation

The level of type 1 pili expression is known to vary with culture conditions. Optimal type 1 pili induction conditions are static growth at 37°C for 24 hours followed by subculture and a further 24 hour static incubation at 37°C (often referred to as 2x24 cultures) (Greene et al., 2015, Zhang et al., 2016). However, for biofilm assays in this thesis, bacterial cells were prepared by overnight growth in 2ml LB, at 37°C in a shaking incubator.

In order to confirm that the culture conditions used to prepare cells for biofilm assays did not prevent the expression of type 1 pili, haemagglutination assays (HA) titres were carried out as described in Materials and Methods section 2.3). Mannose sensitive HA titres are the standard functional test for type 1 pili (Hultgren et al., 1986, Hultgren et al., 1990, Salit and Gotschlich, 1977a). Type 1 pili have been shown to bind to mannose like residues on guinea pig red blood cells and cause haemagglutination – this haemagglutination is mannose sensitive, as mannose competitively binds the FimH adhesin (Salit and Gotschlich, 1977a). Briefly, in HA titre experiments bacteria are serially diluted then exposed to a standardised concentration of guinea pig red blood cells in microtitre plates (full details in Materials and Methods section 2.3). Each strain is given a HA titre value of 0-12 depending on the first well, or highest bacterial concentration) to show no haemagglutination.

Haemagglutination (HA tire of 8) was observed with WT UTI89 cells after overnight shaken growth at 37°C (Figure 3.11a) indicating that type 1 pili were functional in this strain. With UTI89Δ*fim* on the other hand, no haemagglutination (Figure 3.11a) was observed, confirming that type 1 pili are not functional in this strain. The haemagglutination phenotype was successfully rescued by complementation, using the control strain, *fim* complement (plasmid complementation of the full *fim* operon in UTI89Δ*fim*); *fim* complement achieved a HA titre of 8, equal to that of WT UTI89 (Figure 3.11a). As a further control, the same HA titre assay was performed alongside in the presence of mannose. As previously mentioned, the addition of mannose inhibits type 1 pili mediated haemagglutination of guinea pig red blood cells by competitively binding the FimH

adhesin. In the presence of mannose, haemagglutination was not observed with WT UTI89, nor *fim* complement confirming that the haemagglutination by these strains was mannose sensitive. In summary, HA titres confirmed that type 1 pili are expressed in type 1 pili proficient UTI89 strains when grown in shaking overnight cultures at 37°C prior to biofilm formation in the CV assay (as described in Materials and Methods section 2.2.1).

This data also supports previous data in this chapter (Figure 3.1, Figure 3.2 and Figure 3.3) that biofilm formation at 37°C is type 1 pili independent. Type 1 pili are clearly shown here to have been expressed in WT UTI89 at 37°C before biofilm inoculation. However, despite UTI89Δ*fim* biofilm formation occurring at WT levels, no haemagglutination was observed with this strain, confirming that type 1 pili are not functional in this strain and they therefore cannot be responsible for the increased biofilm formation at 37°C.

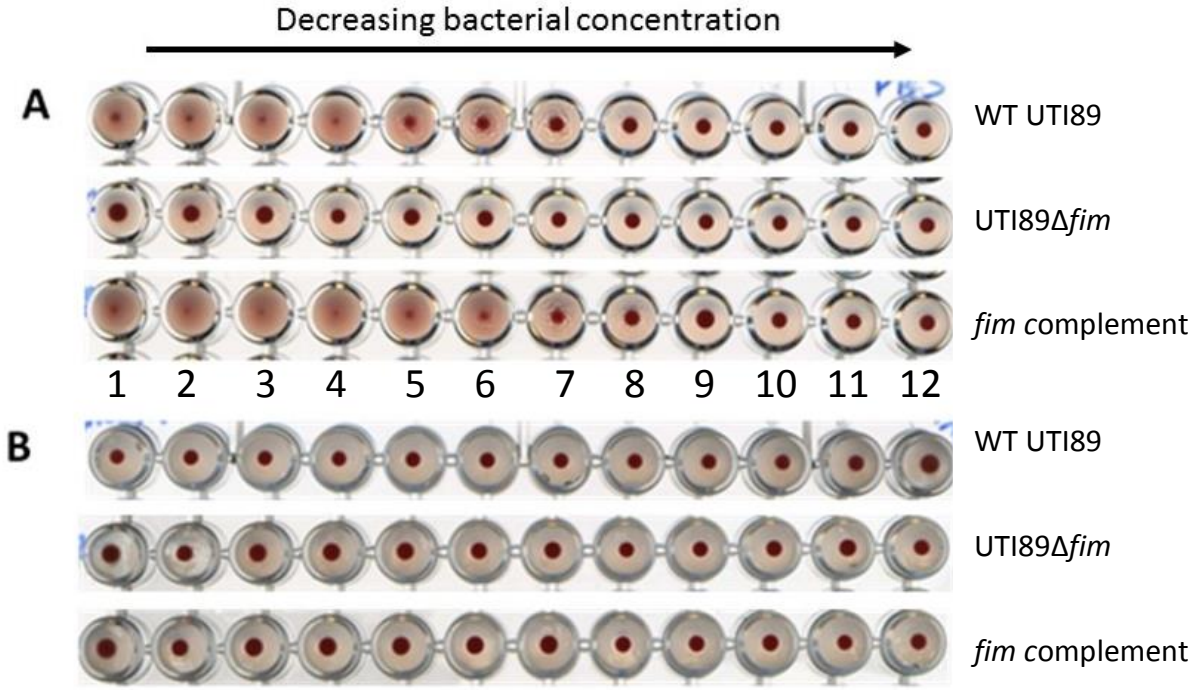


Figure 3.11 Haemagglutination titres of UTI89 wild type and mutant strains prior to biofilm formation.

Bacteria were serially diluted in a microtitre plate and then exposed to a standardised concentration of guinea pig red blood cells. The HA titres in panel A indicate the presence of functional type 1 pili in both the WT UTI89 and the *fim* complement strain, whilst confirming their absence in UTI89Δ*fim.* In panel B – the use of mannose in the assay confirmed that the haemagglutination in (a) was in fact type 1 pili mediated by competitively binding the FimH adhesin and preventing haemagglutination.

3.4.7 Assessing the fitness of WT UTI89 and UTI89Δ*fim* strains at 25°C and 37°C

In order to discount the possibility that a fitness advantage or defect may be the cause of the biofilm phenotype observed (type 1 pili independent biofilm formation at 37°C), planktonic growth curves were obtained for WT UTI89, UTI89Δ*fim* and the *fim* complement strain at both 25°C and 37°C. Growth curves were generated by sequential OD_{600} measurement over a period of 23 hours (as described in Materials and Methods section 2.1.2). Conditions were designed to mimic those in the microtitre plate as closely as possible: strains were handled in the same way prior to inoculation; the same concentration of cells was used as the inoculum and static growth was used (aside from a brief agitation of the cells prior to each measurement).

No significant differences between OD_{600} measurements of WT UTI89 and UTI89Δ*fim* were observed at any time point at both 25°C and 37°C (Figure 3.12). At 25°C OD_{600} measurements for *fim* complement do not differ significantly from WT UTI89 for the first 17 hours. However, OD_{600} measurements for this strain are significantly lower that WT UTI989 between hours 18 ($p = 0.0488$) and 23 ($p = 0.019$) as this strain reaches the stationary phase (Figure 3.12). At 25°C OD_{600} measurements differ more markedly to WT UTI89 over the course of the experiment, aside from the first hour where the cell density is not significantly different to WT UTI89, all subsequent OD_{600} measurements are significantly lower than that of WT UTI89 indicating slower growth and a lower final OD_{600}.

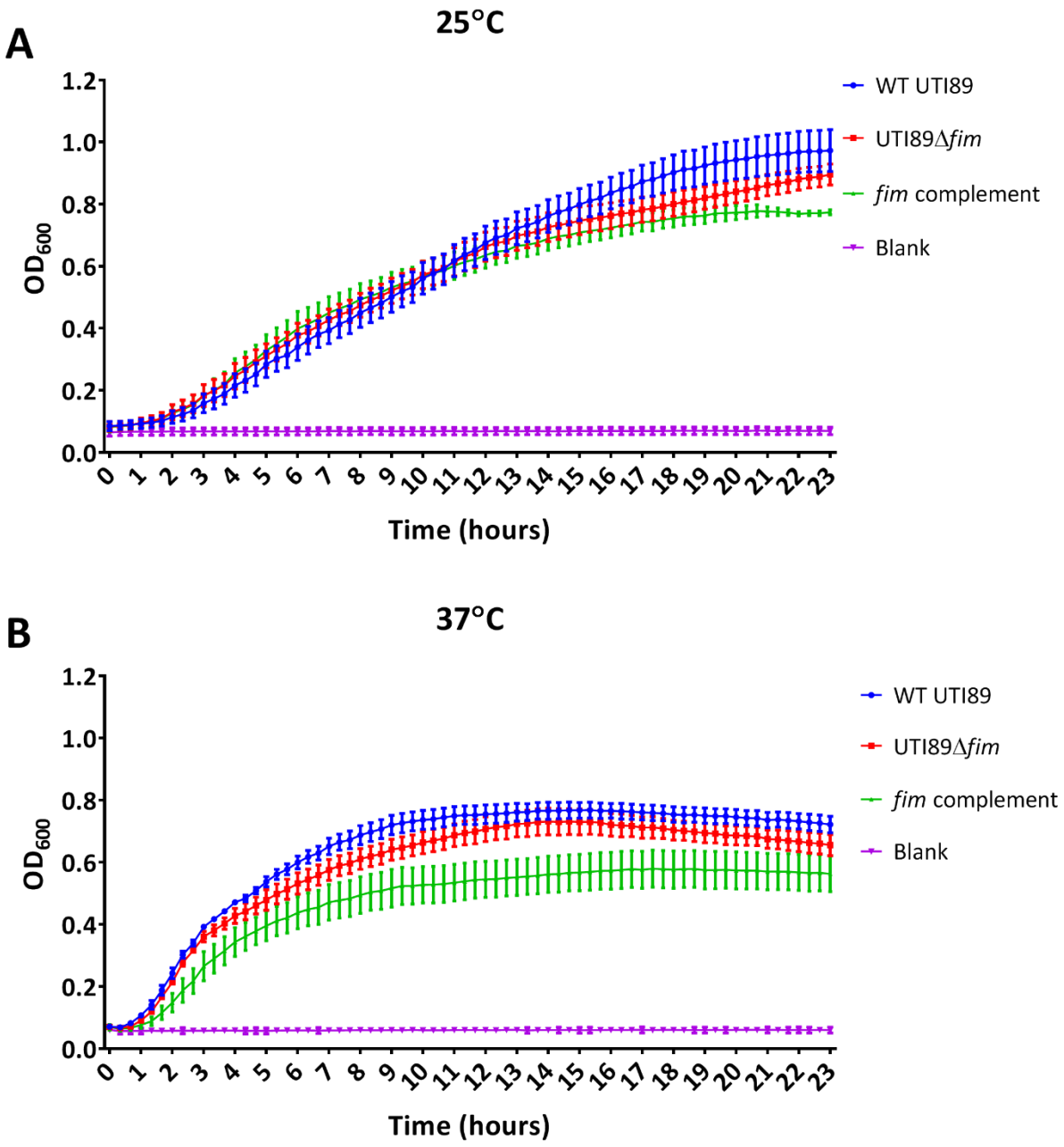


Figure 3.12 Growth curves for wild type UTI89, UTI89Δ*fim* and the respective *fim* complement strain

Growth curves for WT UTI89, UTI89Δ*fim* and the respective *fim* complement strain were generated in triplicate for each stain in LB media over the course of 23 hours using a the Bioscreen C machine (Oy Growth Curves Ab Ltd). Sequential OD_{600} measurements were taken every 20 minutes at both 25˚C (panel A) and 37˚C (panel B). Data shown is the mean OD_{600} value from three individual experiments at each temperature and error bars represent ±1 SEM.

3.5 Discussion

Biofilm formation is reported to be dependent on type 1 pili in an *in vitro* CV assay at 25°C (Lo et al., 2014, Hadjifrangiskou et al., 2012, Pratt and Kolter, 1998). However, an initial observation was made in the Chen lab (Majid Eshaghi & Swaine Chen, personal communication, September 2013), that biofilm formation occurred in the absence of type 1 pili, in the same microtitre biofilm assay, at 37°C. This Chapter aimed to validate and characterise this initial observation and to ascertain the importance of type 1 pili for *in vitro* biofilm formation at both 25°C and 37°C.

Phenotypic characterisation of the clinical UPEC strain UTI89 and several type 1 pili mutant strains revealed differential biofilm formation at varying temperatures. Biofilm formation was shown genetically (Figure 3.1 and Figure 3.2) to be type 1 pili dependent at 25°C, whilst being type 1 pili independent at 37°C. This was confirmed by chemical inhibition. Specific inhibition of the FimH adhesin by mannose reduced type 1 pili dependent biofilm formation at 25°C. However, at 37°C mannose was not able to reduce biofilm formation confirming that at this temperature biofilm formation did not require type 1 pili (Figure 3.3). When *in vitro* biofilm formation by UTI89 was tested at temperatures ranging from 25°C to 37°C, the type 1 pili independent biofilm formation phenotype observed in this chapter was seen to occur specifically at 37°C (Figure 3.4) suggesting that this phenotype may be clinically relevant as this is the physiological temperature at which UTI and CAUTI occur.

Growth curves confirmed that neither the decrease in biofilm formation of UTI89Δ*fim* compared to WT UTI89 at 25°C, or the lack of attenuation relative to WT observed at 37°C, are caused by a growth phenotype as growth curves for these two strains did not differ over the course of 23 hours at either temperature.

Type 1 pili were shown to be functional prior to biofilm formation in WT UTI89 and *fim* complement but not in UTI89Δ*fim* using haemagglutination assays, the standard functional test to confirm type 1 pili presence. However, in order to exclude the possibility that the

haemagglutination observed in type 1 pili positive strains was caused by another factor and to strengthen these results, Western blots could be performed in order to confirm the expression of type 1 pili components.

In order to determine a possible mechanism for the type 1 pili independent biofilm phenotype observed at 37°C, the roles of three other well-known biofilm factors for UPEC, curli, flagella and S pili were tested. All three factors were found not to be largely responsible for the type 1 pili independent biofilm formation of UTI89 observed *in vitro* at 37°C (Figure 3.5*,* Figure 3.6 and Figure 3.7). Whilst deletion of the S pilus did result in a small reduction of biofilm formation it does not substantially reduce biofilm formation relative to WT UTI at 37°C and is therefore unlikely to be the main factor involved in type 1 pili independent biofilm formation at 37°C. The slight reduction observed with this strain is likely due to the inverse relationship in expression between type 1 pili and S pilus that has been previously described (Greene et al., 2014, Kostakioti et al., 2009) whereby S pilus is increased in the absence of type 1 pili. Previous studies have shown that there may be hundreds of factors involved in biofilm formation (Hadjifrangiskou et al., 2012). The list of factors tested here is by no means exhaustive but does show that several of the most well-established virulence factors for UPEC do not play a role in type 1 pili independent biofilm formation at 37°C and the mechanism behind this biofilm formation remains unknown. Further work is required to elucidate what causes type 1 pili independent biofilm formation at 37°C and will be addressed in Chapter 5: Genomic approaches to biofilm phenotype characterisation.

The same type 1 pili independent phenotype was observed at 37°C with clinical UPEC isolate CFT073 but not with the laboratory strain MG1655 (Figure 3.8 and Figure 3.9). Type 1 pili deficient MG1655 were unable to form biofilms at either 25°C or 37°C, this is perhaps unsurprising as MG1655 is a non-pathogenic laboratory *E. coli* strain.

The biofilm formation phenotype observed for UTI89 at 37°C also appears to be unaffected by the abiotic adherence material used in the assay, with the same results observed using PVC and polystyrene plates (Figure 3.10). The material used for the majority of *in vitro* assays, PVC, is not

necessarily the most relevant to CAUTI as most catheters are made of silicone. Although the possibility remains that this phenotype may only be observed on PVC and polystyrene, it can be postulated that type 1 pili independent biofilms form on a range of abiotic surfaces.

As type 1 pili are well known to be a crucial virulence factor for UTI, it has also been assumed that they play a role in CAUTI and adherence to catheters. However, biofilm formation on an abiotic surface was shown to be independent of type 1 pili at 37°C. Arguably, this is a more physiologically relevant temperature. The ability to change the mode of biofilm formation in response to temperature is extremely interesting in terms of CAUTI as the temperature varies along the length of the catheter from 37°C at the tip down to ambient temperature (around 25°C) at the collection bag. This switch in biofilm mode may be utilised by the bacteria in order to gain a foothold of infection in a particular condition and then spread between areas utilising the change in biofilm formation mode. *In vivo* characterisation of this phenotype using a model for UTI and CAUTI will help to examine the *in vivo* relevance of the type 1 pili dependent biofilm formation at 37˚C *in vitro* (see Chapter 4) and to establish any correspondence between the *in vitro* and *in vivo* models.

Chapter 4: *In Vivo* Characterisation of Type 1 Pili Independent Biofilm Formation

4.1 Introduction

The pathogenesis of UTI89 has been extensively studied. This UPEC strain has been shown to form IBCs *in vivo,* in a mouse model for UTI, and *in vitro,* in human bladder epithelial cell lines, (Wright et al., 2007, Rosen et al., 2007, Martinez et al., 2000). IBCs have also been identified in the urine of UTI patients (Rosen et al., 2007) confirming the relevance of these structures to human infection. The process of IBC formation has previously been shown to be dependent on type 1 pili with UTI89 cells deficient in type 1 pili being unable to cause infection *in vivo* (Wright et al., 2007). Type 1 pili have also been shown to be essential for biofilm formation in an *in vitro* CV model at 25°C both in published literature and previously in this thesis (Chapter 3) (Hadjifrangiskou et al., 2012, Lo et al., 2014, Pratt and Kolter, 1998). This corresponds with the published *in vivo* infection data. Despite this strong data concerning type 1 pili, previous data in this thesis has also shown that biofilm formation in the same *in vitro* biofilm model is type 1 pili independent at 37°C, contrary to that at 25°C. This suggests that type 1 pili deficient UPEC strains may be able to form biofilm on abiotic surfaces at physiological temperatures *in vivo.* Furthermore, a murine model for CAUTI has shown *E. faecalis* to be pathogenic in the presence of a catheter implant despite being unable to cause infection in naive mouse bladders (Guiton et al., 2012, Guiton et al., 2010). It was therefore hypothesised that type 1 pili deficient UTI89 may also be able to cause infection in the murine CAUTI model, corresponding to the *in vitro* data presented in Chapter 3.

So far, this thesis has shown a correlation between temperature, biofilm formation mechanism and the requirement for type 1 pili *in vitro*. Temperatures of 25°C and 37°C correspond with those in human CAUTI and characterisation studies have shown that type 1 pili independent biofilms form of several different abiotic surfaces. This raises the question of whether type 1 pili deficient UPEC are able to colonise catheters and cause infection in a murine model for CAUTI.

Therefore, this chapter explores the *in vivo* relevance of the type 1 pili independent *in vitro* biofilm phenotype previously described in Chapter 3.

4.2 Aims

To establish the relevance of previous *in vitro* biofilm studies (See Chapter 3) to mammalian disease by testing the ability of type 1 pili deficient UPEC to cause infection in a murine model for CAUTI.

4.3 Hypotheses

1. Type 1 pili deficient UTI89 are able to cause infection and colonise catheters in a mouse model for UTI.
    - As type 1 pili are not required for biofilm formation at 37°C *in vitro* (as shown in Chapter 3), they are also not required to cause infection and colonise catheter implants *in vivo* in a murine CAUTI model.
2. The same is true for Pyelonephritis strain CFT073 which is also able to cause infection and colonise catheters in a mouse model for CAUTI.

4.4 Results

4.4.1 Type 1 pili are not essential for bladder infection and catheter colonisation by UPEC strains UTI89 and CFT073 in catheterised mice

Using a murine CAUTI model previously optimised for *E. faecalis* (Guiton et al., 2010, Conover et al., 2015), the *in vivo* relevance of type 1 pili independent biofilm formation was assessed. Briefly, groups of naïve mice or mice with a silicone catheter implant in their bladder were challenged with an inoculum of either WT UTI89 or UTI89Δ*fim.* The mice were sacrificed at 24 hours post infection (hpi), their bladders and kidneys harvested and catheters recovered. Harvested bladders and kidneys were homogenised in 1 ml and 800 µl of cold PBS respectively and homogenised and catheters were sonicated in 1 ml PBS. The bacterial load of the homogenates and sonicates were then determined by CFU enumeration (see Materials and Methods section 2.4.2 for full details). The same experiment was carried out using *E. faecalis* strain OG1RF alongside the UPEC strains in order to act as a control for the model system, as it has previously been well characterised with this strain (see Materials and Methods section 2.4 for full experimental details). In line with previous reports, low bladder titres were observed for *E. faecalis*, however bladder titres were significantly increased by around 1×10^4 CFU/ml at 24 hpi in the presence of a catheter implant (p= <0.0001) (Figure 4.1), replicating previously reported results (Guiton et al., 2012, Guiton et al., 2010).

In the standard mouse model for UTI (naïve mice), WT UTI89 was able to consistently colonise the bladder at a high level, with a median of around 5×10^6 CFU/ml (Figure 4.1). However, in the CAUTI model, in the presence of a silicone implant, the median bladder titre significantly decreased by approximately one log to around 5×10^5 CFU/ml (p= <0.0001) (Figure 4.1). In contrast, UTI89Δ*fim* exhibits low bladder titres (median ~ 3×10^1 CFU/ml) in the standard UTI model (Figure 4.1) in line with previously reported data (Wright et al., 2007). In the CAUTI model however, UTI89Δ*fim* displayed a significant increase in bladder titres of around one log in catheterised mice compared to UTI89Δ*fim* in naïve mice (from 3×10^1 CFU/ml to 5×10^2 CFU/ml, (p= 0.0010)), with multiple mice

showing even higher levels of infection in catheterised mice of up to 5-6 log higher compared to naïve mice (up to 2×10^7 CFU/ml) (Figure 4.1).

Previous data from this thesis have indicated that the *in vitro* biofilm phenotype identified and characterised for UTI89 is the same for another UPEC strain, CFT073 (a prototypic pyelonephritis strain). In Chapter 3, biofilm formation by CFT073 at 25°C was shown to also be dependent on type 1 pili whilst being type 1 pili independent at 37°C (Figure 3.8). As CFT073 displayed the same phenotype to UTI89 in the *in vitro* CV model, it was hypothesised that the same may be true for the *in vivo* CAUTI model. Further testing of the correlation between the *in vitro* biofilm assay and the CAUTI mouse model was carried out by assessing the ability of CFT073 and a respective *fimH* mutant to cause infection in both the UTI and CAUTI murine models.

WT CFT073 exhibited a median bladder titre of 5×10^5 CFU/ml in naïve mice, this was significantly higher, by 1×10^5 CFU/ml (p = <0.0001) than mice infected with CFT073Δ*fimH* supporting previous reports that functional type 1 pili are required for CFT073 infection in a mouse model for UTI (Gunther et al., 2001, Gunther et al., 2002). In the presence of a catheter implant the bladder titres for WT CFT073 did not differ significantly to those in naïve mice. However, CFT073Δ*fimH* showed a very slight but statistically significant increase in bladder titre in catheterised mice compare to naïve mice (p = 0.0064). Although less pronounced than with UTI89Δ*fim* this increase in bladder titres suggests a role for an alternative mechanism of infection other than type 1 pili in the presence of an implant. The difference between the effect of a catheter implant on infection with UTI89Δ*fim* and CFT073Δ*fimH* compared to their respective WT strains could be a result of the number of mice used for each group (n=20 CFT073, n=40 UTI89).

The majority of implanted mice (77%) retained their catheter implants in the bladder or urinary tract until the time of sacrifice (24 hpi). However, this percentage is lower than the implant

retention rate reported by Guiton et al. (2010) of 95% at 24 hpi. Those mice that lost[1] the catheter implant during the course of the experiment were treated as failed experiments and were not included in subsequent data analysis.

When mice were sacrificed at 24 hpi, catheter implants were also recovered from the bladders of catheterised mice. The bacterial load of each catheter implant was assessed (again using *E. faecalis* as a control for the model system). Briefly, catheter implants were placed in 1 ml sterile PBS and then sonicated followed by vortexing in order to dislodge adhered bacteria, the bacterial load of each sonicate was then determined by CFU enumeration (see Materials and Methods sections 2.4.5 and 2.4.6 for full details).

Catheter implants from mice infected with WT UTI89 have a mean bacterial load of $4x10^5$ CFU/ml, and whilst the level of colonisation is significantly lower (p = <0.0001) in the absence of functional type 1 pili, UTI89Δ*fim* had the ability to colonise catheter implants with a mean bacterial load of 6×10^2 CFU/ml. The bacterial load on the catheter with WT CFT073 was significantly higher than CFT073Δ*fim* (p = 0.0018) (Figure 4.2). In general, increased bladder titres in the type 1 pili deficient strains seemed to be linked to the presence of a catheter in implanted mice, with higher bladder titres being observed in mice with higher bacterial loads as presented by matched bladder and catheter titres in Figure 4.3, in particular panels b and d.

It has been previously reported that the presence of a catheter causes increased ascension of *E. faecalis* infection to the kidneys in the CAUTI model compared to the UTI model (Guiton et al., 2012, Guiton et al., 2010). This data was recapitulated with *E. faecalis* and a significant increase in the bacterial load of mouse kidneys was observed in the presence of a catheter implant (Figure 4.4). However, despite higher bladder titres in catheterised mice, neither wild type UTI89 nor UTI89Δ*fim*

[1] A lost catheter or implant refers to mice that were implanted with a 5mm piece of silicone tubing prior to infection but from which an implant was not recovered from the bladder or lower urinary tract 24 hpi, upon sacrifice.

exhibited any significant increase in the bacterial load of kidneys in the presence of the catheter (Figure 4.4) (significance values for all comparisons are presented in Appendix A1). The same was also true for CFT073 infection in this mouse model for CAUTI. Neither WT CFT073 nor CFT073Δ*fimH* showed any increase in kidney titres in the presence of a catheter. WT CFT073 however, irrespective of a catheter implant, did infect kidneys to much higher levels than CFT073Δ*fimH* and UTI89 strains. This is to be expected as CFT073 is a clinical pyelonephritis (kidney infection) strain that has been previously shown to infect mouse kidneys in a type 1 pili dependent manner (Bahrani-Mougeot et al., 2002, Gunther et al., 2001, Gunther et al., 2002).

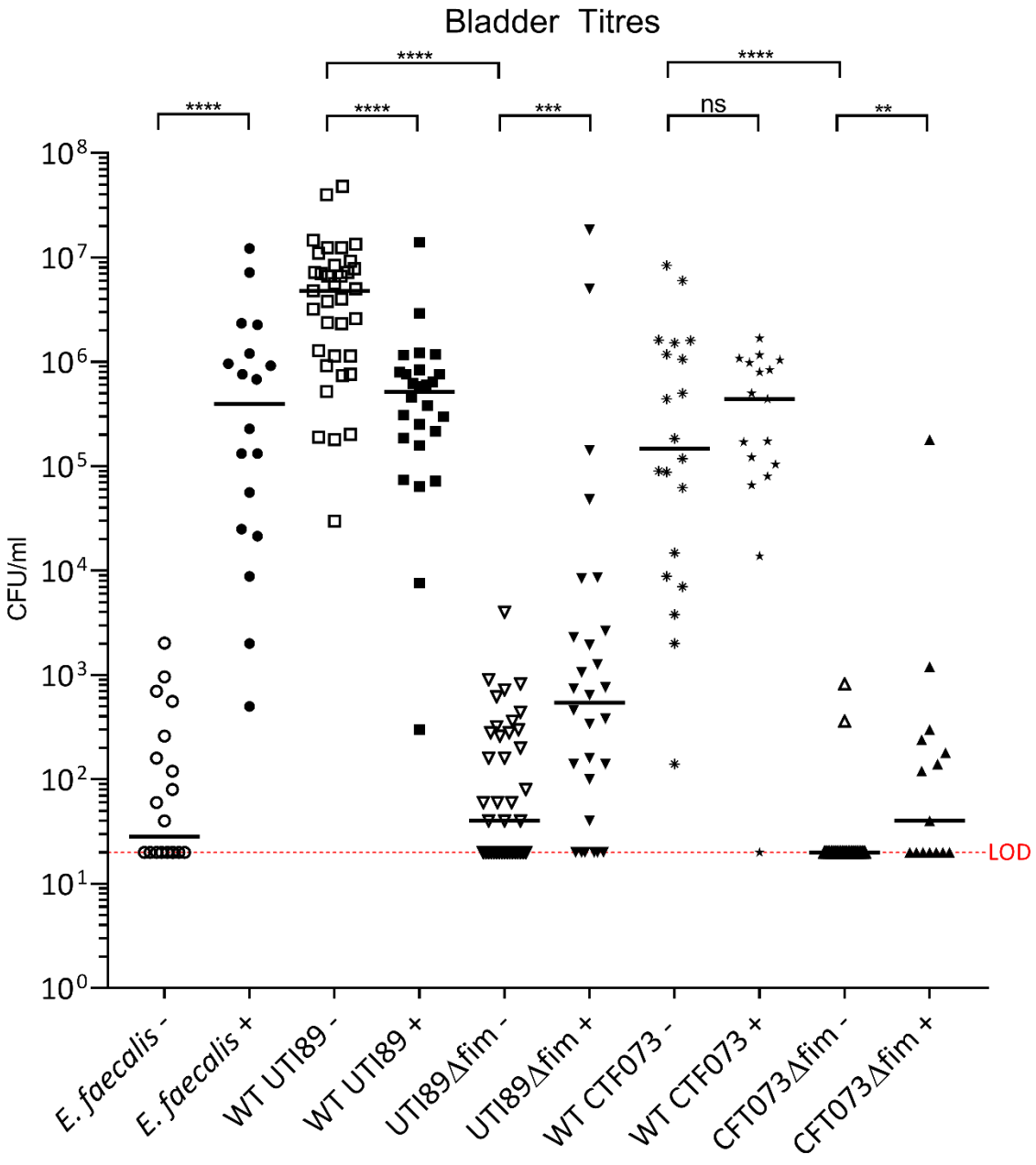


Figure 4.1 Bladder titres for UTI89 and CFT073 and their respective type 1 pili mutant in a mouse model for UTI and CAUTI.

Column scatter plot showing the bacterial load of homogenised bladders from infected mice, either with (+) or without (-) a catheter implant, at 24hpi. The Limit of detection for CFU enumeration was 20 CFU/ml. Two-tailed Mann-Whitney U test was performed to compare pairs of groups in order to determine significance. *p* values ≤0.05 were considered significant. Asterisks denote level of significance * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. *p values* for all possible comparisons are shown in Appendix A.1. Data in this figure is from 8 individual experiments. Each data point represents one mouse and each group consists of data from at least 3 individual experiments with at least 5 mice per group, per experiment.

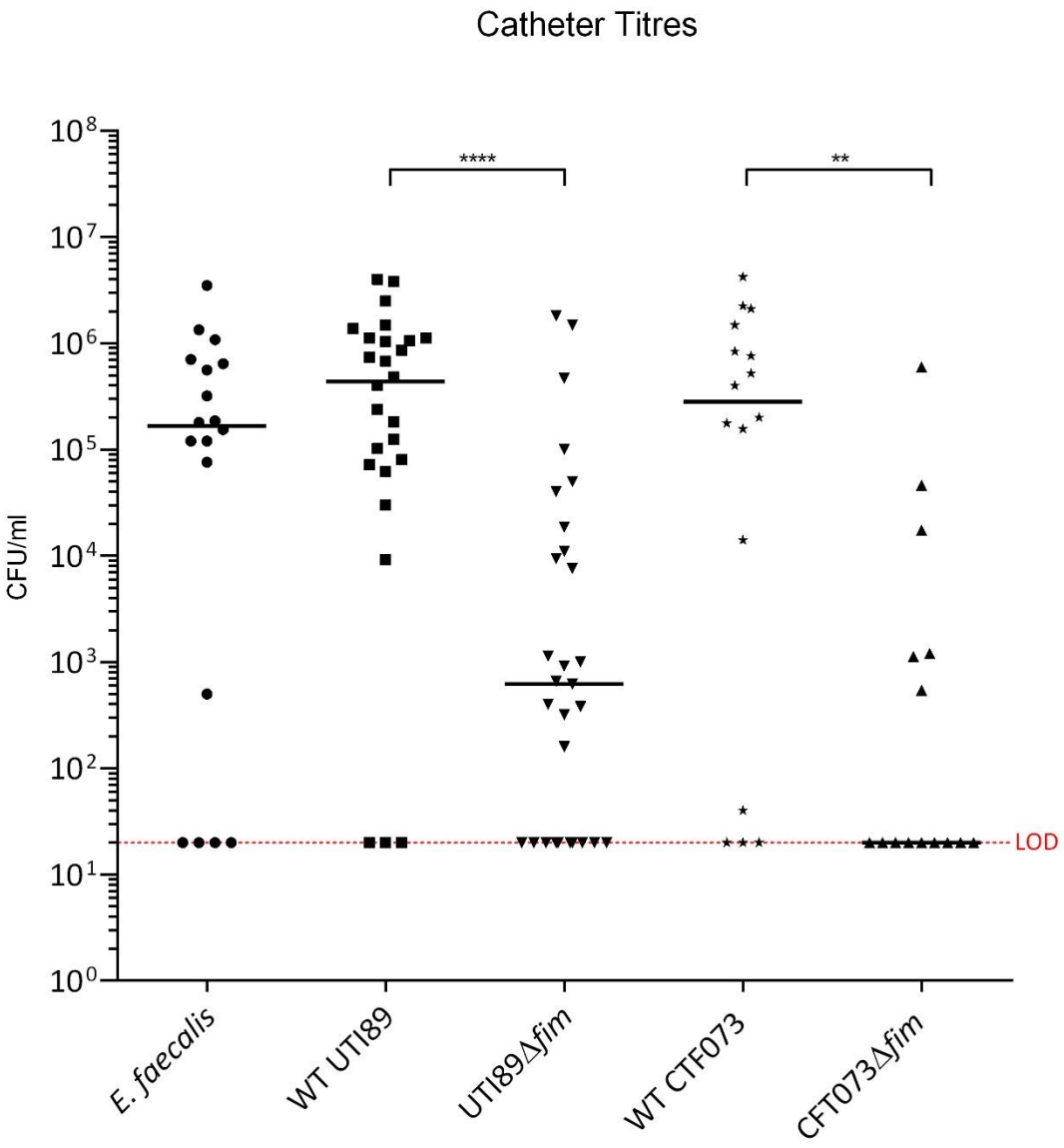


Figure 4.2 Type 1 pili deficient UTI89 are able to colonise catheter implants in a mouse model for CAUTI.

Column scatter plot showing the bacterial titres from catheters recovered from the bladders of infected mice at 24 hpi. The limit of detection (LOD) for this CFU enumeration was 20 CFU/ml. The two-tailed Mann-Whitney U test was performed to compare pairs of groups in order to determine significance. *p* values ≤0.05 were considered significant. Asterisks denote level of * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Data in this figure is from 8 individual experiments. Each data point represents one catheter from one mouse and each group consists of data from at least 3 individual experiments with at least 5 mice per group, per experiment.

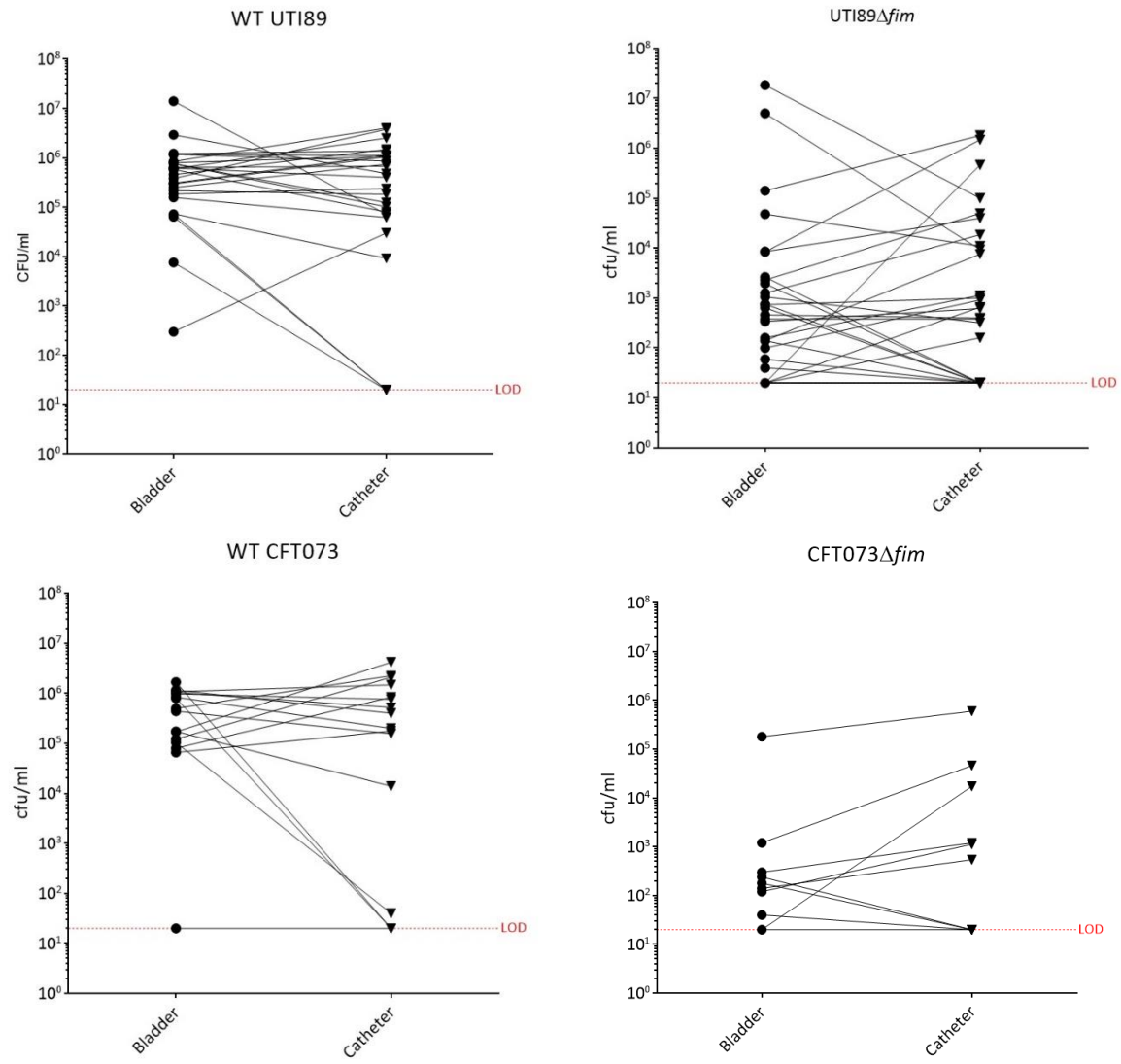


Figure 4.3 Matched bladder and catheter titres

This figure contains the data from the same experiments presented in Figure 4.1 and Figure 4.2 but matching paired bladder and catheter bacterial loads from mice which retained the catheter implants until 24 hpi. Here each pair of dots connected by a line indicates the bladder and catheter titre from one infected mouse. The LOD for this CFU enumeration was 20 CFU/ml. Data is from 8 individual experiments, each group contains information from at least three of those with a minimum of 5 mice per group, per experiment

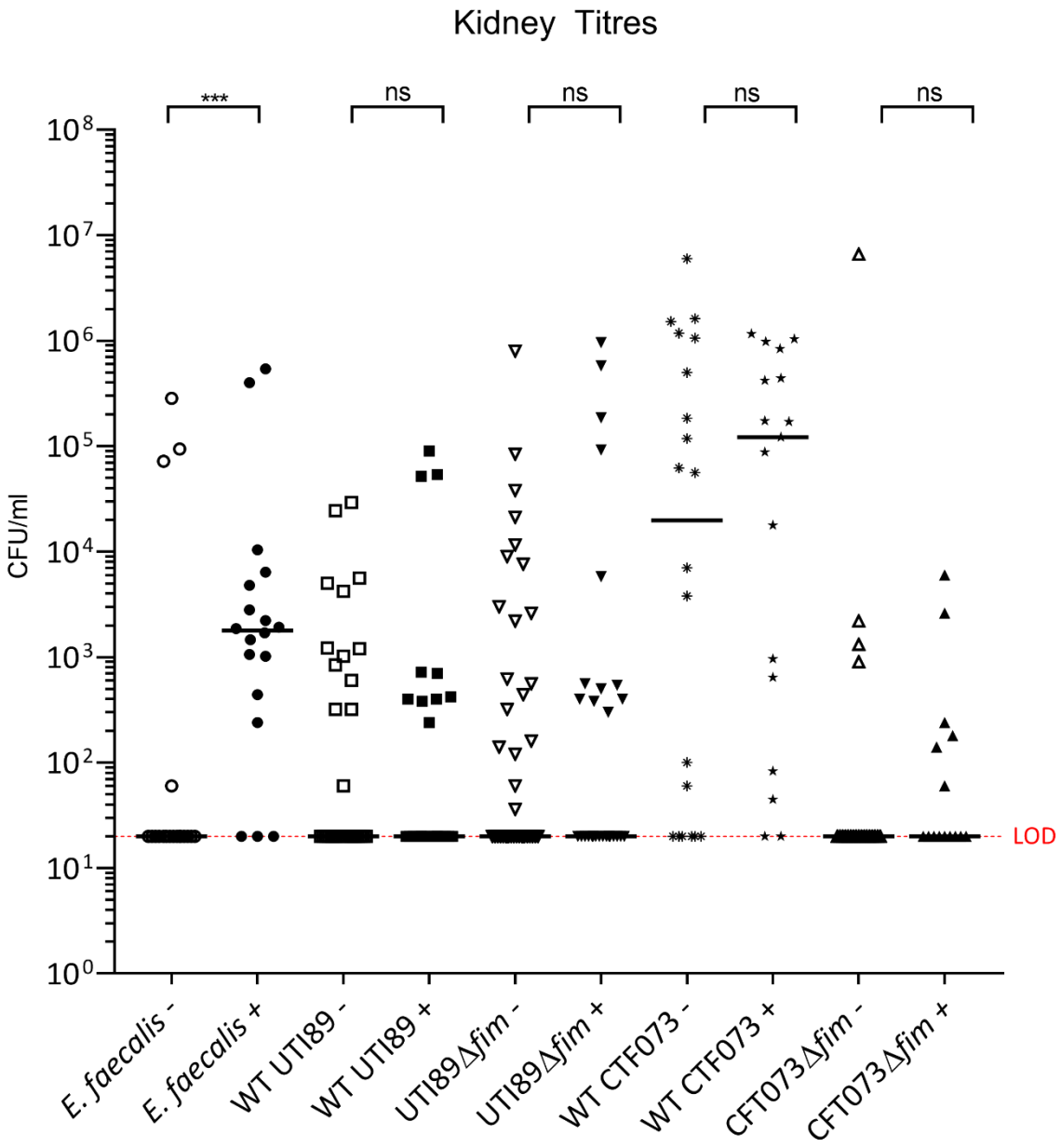


Figure 4.4 Kidney titres for UTI89 and CFT073 and their respective type 1 pili mutant in a mouse model for UTI and CAUTI

Column scatter plot showing the bacterial load of homogenised kidneys from infected mice, either with (+) or without (-) a catheter implant, at 24 hpi. The limit of detection (LOD) for this CFU enumeration was 20 CFU/ml. A two-tailed Mann-Whitney U test was performed to compare pairs of groups in order to determine significance. Asterisks denote levels of significance * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. Aside from *E. faecalis,* no significant difference was observed between any strain tested with (+) or without a catheter (-).

Data in this figure is from 8 individual experiments. Each data point represents the bacterial load of both kidneys from one mouse. Each group consists of data from at least 3 individual experiments with at least 5 mice per group, per experiment.

4.4.2 UTI89Δ*fim* infection in catheterised mice is not IBC mediated

Wild type UTI89 has been shown to form IBCs *in vivo* in mouse bladders (Wright et al., 2007) as well as *in vitro* in human bladder epithelial cell lines (Martinez et al., 2000). These same IBC structures have also been shown to be present in human UTI. BECs containing IBCs have been detected in the urine of patients presenting with UTIs (Martinez et al., 2000, Rosen et al., 2007), confirming the relevance of these well studied structures in human infection. As such, IBCs are considered to be a crucial part of UPEC pathogenesis.

As previously mentioned, in the mouse model for UTI it is clear that type 1 pili are essential for IBC formation by UTI89 (Wright et al., 2007). Using this same model and a murine CAUTI model (see Materials and Methods section 2.4), the IBC forming capabilities of both wild type UTI89 and type 1 pili deficient UTI89 (UTI89Δ*fim)* in both the presence of a catheter implant and in naïve mouse bladders, was assessed. Despite significantly increased bladder titres in catheterised mice infected UTI89Δ*fim* as seen in Figure 4.1, this strain is unable to form IBCs. IBCs are not detected in splayed bladder sections of mice infected with GFP expressing UTI89Δ*fim,* with or without catheter implants. IBCs are only visible in WT UTI89 infected mice (Figure 4.5).

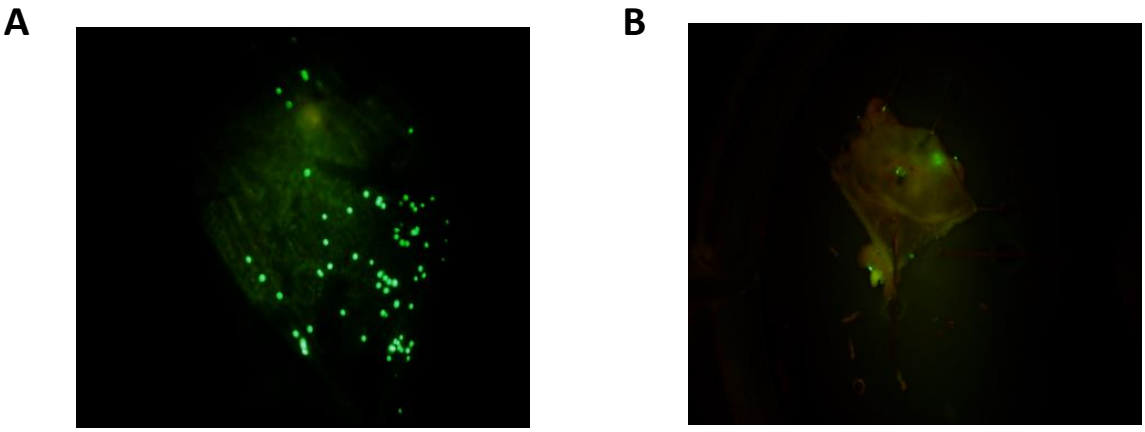


Figure 4.5 Type 1 pili deficient UTI89 are unable to form IBCs in the bladders of both catheterised and naïve mice despite increased bladder colonisation in catheterised mice.

Mice with catheter implants in their bladders and naïve mice were transurethrally infected with WT UTI89 expressing GFP or UTI89Δ*fim* expressing GFP. At 24hpi bladders were harvested, hemisected and splayed prior to fluorescent microscopy in order to visualise GFP expressing IBCs. IBCs are visible in naïve mouse bladders and, to a lesser extent, in the bladders of catheterised mice, but only when functional type 1 pili are expressed (representative images shown). GFP tagged UTI89 can form IBCs in naïve bladders (a) and catheterised bladders to a lesser extent (b), but are not observed at all in either catheterised or naïve bladders in the absence of type 1 pili (not shown).

4.5 Discussion

The major aim of this chapter was to study the ability of UPEC strains, and their respective type 1 pili mutants, to cause infection in a mouse model for UTI and CAUTI. This was to ascertain whether or not there was any correspondence between the *in vitro* type 1 pili independent biofilm formation observed at 37°C (Chapter 3) and *in vivo* infection in order to establish a coherent set of model systems for CAUTI. This thesis has also identified an alternative mechanism, aside from type 1 pili, involved in biofilm formation of UPEC strains UTI89 and CFT073 on abiotic surfaces *in vitro* (see Chapter 3). Briefly, *in vitro* biofilms in a microtitre plate assay at 25°C required type 1 pili, a key virulence factor for UTI, whereas those at 37°C were able to form independent of type 1 pili (Figure 3.1 and Figure 3.3). This phenotype suggests that there may be alternative pathogenesis mechanisms aside from type 1 pili involved in CAUTI, which also involve abiotic surfaces in human infection.

Type 1 pili mediated infection is often modelled *in vitro* using this microtitre plate assay for biofilm formation at 25°C and this corresponds to data from a mouse model of infection that has been shown to be dependent on type 1 pili. The pathogenesis of UTI is well understood and has been shown to be dependent on type 1 pili and because this process has been well characterised, and is generally well understood, it has previously been assumed that the pathogenesis of CAUTIs must overlap with that of regular UTIs although there is comparatively less research in this field. Recent studies by Guiton et al. (2010) and (2012) however, have shown differences in pathogenesis between the two types of infection with *E. faecalis. E. faecalis* is unable to cause infection in a mouse model for UTI, although in a CAUTI mouse model *E. faecalis* is able to take advantage of a catheter implant and cause bladder infection. The ability of UPEC type 1 pili mutants (UTI89 and CFT073) to cause infection was tested in mouse models for both UTI and CAUTI. Despite being dependent on type 1 pili in a standard murine model for UTI, UPEC bladder infection has been shown to be partially type 1 pili independent in a CAUTI model (Figure 4.1). Type 1 pili deficient UPEC strains were also shown to be able to colonise silicone implants in mouse bladders (Figure

4.2) and higher catheter titres seemed to be related to higher bladder titres (Figure 4.3). This increased bacterial load of bladders in implanted mice, coupled with the ability to colonise catheter implants, both in the absence of type 1 pili, suggests a role for an alternative mechanism for *in vivo* biofilm formation in CAUTI. Although the type 1 pili independent effect is less pronounced than *in vitro,* this reflects the *in vitro* data presented in Chapter 3, whereby type 1 pili deficient UTI89 were able to form biofilms independent of type 1 pili at 37°C (the same physiological temperature as that of the mice) on abiotic surfaces (PVC and polystyrene microtitre plates). The relationship between type 1 pili dependence for biofilm formation and the available models for UTI is outlined in Table 4.1. This corresponding set of models will aid further research on the pathogenesis of CAUTI potentially allowing the elucidation of biofilm factors previously overlooked because of the presumed importance of type 1 pili in these infections.

Although the increase in bladder titres observed with UTI89Δ*fim* infection in catheterised mice was a modest increase, it was significant and the increase was to a level that has not been previously observed in standard mouse UTI infection. Particularly for UTI89 in the absence of type 1 pili, bacteria were recovered at levels of up to 1.84×10^7 CFU/ml in individual mice. The presence of a catheter did not increase levels of infection independent of the bacterial strain in a non-specific manner as wild type UTI89 conversely displayed significantly reduced bladder titres in catheterised mice compared to those without a catheter. This decrease was also apparent in infected mouse bladders when imaging IBCs *in situ*. IBCs were plentiful in the bladders of naïve mice but fewer were observed in the bladders of implanted mice. No IBCs were observed in the bladders of mice, either naïve or implanted, infected with type 1 pili deficient UPEC, confirming previous observations that type 1 pili are required for IBC formation. This supports the notion that these bacteria are able to occupy a different niche to those expressing functional pili other than IBCs, for example biofilms on the bladder surface in a presence of a catheter.

Another point of note is that the catheter material used for *in vivo* CAUTI studies (silicone) differs to that of the *in vitro* assay (PVC); however, this project has previously shown that type 1 pili

independent biofilms also form on polystyrene plates at 37°C, indicating that this particular phenotype occurs with a range of adherence materials. The colonisation of silicone implants by both UTI89Δ*fim* and CFT073Δ*fimH* seen in this CAUTI model further supports this idea that the observed phenotype forms on various adherence materials, increasing its relevance to CAUTI, although definitive biofilm formation has not been tested on all abiotic surfaces.

The presence of a catheter implant didn't not increase the infectivity of type 1 pili deficient UPEC strains in the kidney as has been previously reported for *E. faecalis* infection. However, an increase in kidney titres was observed at later time points for *E. faecalis* (Guiton et al., 2010), suggesting that a more pronounced UPEC kidney infection may be observed at later time points.

Uncomplicated UTI have been extensively studied and the strong dependence of type 1 pili for infection has been extremely well documented, however the exact pathogenesis of CAUTI is still relatively poorly understood. This thesis has shown that type 1 pili are not in fact required for infection in a mouse model for CAUTI, despite being essential in uncomplicated UTI in mice, suggesting a role for an alternative mechanism of infection, aside from type 1 pili in CAUTI. The correspondence of this phenotype to the previously observed *in vitro* biofilm phenotype of type 1 pili independent biofilm formation at 37°C is extremely valuable in the study of CAUTI. A complete set of corresponding model systems for the study of CAUTI will aid the further study and understanding of CAUTI, as is currently the case for UTI.

4.6 Further Work

In order to further this study of type 1 pili independent infection in relation to CAUTI, the ability of UPEC to cause infection in a mouse model for CAUTI in the absence of functional type 1 pili could be tested at later time points. This chapter has established a modest but significant increase in bladder titres for type 1 pili deficient UPEC in this *in vivo* model compared to in naïve mice. It is possible that this phenotype may become more pronounced with an increased length of infection with the catheter potentially being able to support persistent infection in these otherwise

'non-pathogenic' strains. This may also lead to a more significant effect on kidney infection cause by the presence of an implant as has been suggested for other bacteria up to 5 days post infection (Guiton et al., 2010).

Up to this point, the data show that type 1 pili deficient UPEC are able to form biofilms *in vitro* at 37°C and also infect bladders and colonise catheter implants *in vivo* in a mouse model for CAUTI. Although having described this type 1 pili independent biofilm formation *in vitro* and confirming the relevance of this phenotype *in vivo*, the mechanisms behind biofilm formation and pathogenesis however, have not yet been determined. Further characterisation of the phenotype will be extremely useful in order to elucidate potential factors involved in biofilm formation *in vitro* with a view to better understanding the pathogenesis of CAUTI *in vivo*, and potentially in human infection. High throughput genomic screening is a very useful tool that can be applied to this purpose and is discussed in Chapter 5: Genomic approaches to biofilm phenotype characterisation.

Table 4.1 The relationship between *in vitro* and *in vivo* UTI, CAUTI and biofilm models and type 1 pili dependence

	Type 1 pili dependent	**Type 1 pili independent**
In vitro	Biofilm formation at 25°C in microtitre plate assay	Biofilm formation at 37°C microtitre plate assay
In vivo	Standard UTI model IBC mediated infection Bladder titres	CAUTI (implant) model Bladder and catheter titres

Chapter 5: Genomic Approaches to Biofilm Phenotype Characterisation

5.1 Introduction

Having identified and phenotypically characterised a difference in biofilm formation, whereby UPEC strain UTI89 is able to form biofilms independent of type 1 pili at 37°C whilst being dependent on type 1 pili at 25°C, further genetic characterisation and study was required in order to gain insight into this biofilm phenotype. A previously reported transposon screen indicated that there may be as many as 170 genes involved in UTI89 biofilm formation *in vitro* (Hadjifrangiskou et al., 2012), and having now established that several well-known biofilm factors are not required for biofilm formation at 37˚C (type 1 pili, curli, flagella and S pili) an unbiased genome wide approach is required in order to elucidate any genes that may be required for type 1 pili independent biofilm formation at 37°C.

Several technologies that allow unbiased high-throughput genetic phenotypic screening have recently emerged. Transposon sequencing (Tn-seq) is one such approach that would be useful in this instance. These approaches combine the principle of a traditional phenotypic transposon screen with next generation sequencing (NGS) allowing the extremely high-throughput phenotypic screening of a whole bacterial genome. Transposon directed insertion site sequencing (TraDIS) is a variation of this technique. TraDIS (Langridge et al., 2009) is one of several Tn-seq methods that have recently been developed to study the genes involved in a particular bacterial function. These Tn-seq methods combine the principle of a traditional transposon mutagenesis with high-throughput NGS and allow the study of an entire genome simultaneously, removing the need for manual, labour intensive screens that use a gene by gene approach. Aside from TraDIS, other examples of transposon sequencing methods include Tn-seq (van Opijnen et al., 2009) and INSeq (Goodman et al., 2009), which both use a Mariner transposon, and HITS (Gawronski et al., 2009) which, like TraDIS, uses a Tn5 transposon. Originally developed by the Wellcome Trust Sanger

Institute and used to simultaneously assay every gene in S*almonella* Typhimurium (Langridge et al., 2009), TraDIS, has since been adapted to evaluate the genes required by various different bacteria in different test conditions (Phan et al., 2013, Luan et al., 2013, Byrne et al., 2014, Ates et al., 2015, Eckert et al., 2011, Subashchandrabose et al., 2013).

TraDIS was selected for use in this thesis for two complementary reasons: firstly, it can be used with any transposon; secondly, it allowed the use of a commercially available transposon that had previously been used with success in UTI89 (Hadjifrangiskou et al., 2012). The TraDIS methodology has also previously been used successfully in both UTI89 (Subashchandrabose et al., 2013) and other *E.coli* strains (Eckert et al., 2011, Phan et al., 2013).

To perform TraDIS a complex library of transposon mutants is created using insertional mutagenesis with a Tn5 transposon. This library of transposon mutants is passaged in screening conditions specific to the bacterial function being studied. In this thesis for example, the aim was to study the genes involved in biofilm formation at various temperatures and so the transposon mutant library generated was grown up in a microtitre biofilm assay at 25°C and 37°C. After this passage, the DNA is extracted from the cells and the transposon insertion junctions present in this DNA were sequenced. The sequenced junctions can then be mapped back to the reference genome for the bacteria in question. Genes that are essential for the test condition will not be detected in sequencing data. This is due to the fact that cells containing a mutated essential gene (transposon insertion), will be outcompeted in the specific test conditions by those cells in the mutant library population that do not contain disrupting transposon insertions in this gene. Genes which have much fewer, or no mapped reads, are therefore considered to be essential for the conditions tested.

The aim of this chapter was to use TraDIS in order to screen the UTI89 genome for genes essential for biofilm formation at 25°C and 37°C in a high-throughput manner. This was to allow the further understanding of the type 1 pili independent biofilm formation identified previously in this

thesis, with a view to furthering the understanding of UPEC pathogenesis in relation to CAUTI (a relatively less studied area compared to UTI (Jacobsen et al., 2008))

5.2 Aims

The aim of this chapter is to genetically characterise the variation in biofilm formation mechanisms at different temperatures using an unbiased, high-throughput genetic screen. Transposon directed insertion site sequencing (TraDIS) will be utilised with a view to identifying causative genes responsible for biofilm formation at both 25°C and 37°C *in vitro* in the microtitre plate assay used previously in this thesis.

5.3 Hypothesis

1. At 37°C biofilm factors other than type 1 pili, curli, flagella and S pili are crucial for UTI89 biofilm formation and can be identified by combining a traditional transposon screen with NGS technologies.

5.4 Results

5.4.1 Generation of a library of UTI89 transposon mutants

In order to study genes essential for UTI89 growth and biofilm formation a complex mutant library was generated by random transposition of a Tn5 transposon into WT UTI89 cells. This mutant library was created by electroporation of the EzTn5 transposon from epicentre into UTI89 WT cells (SLC-6) as described in Materials and Methods section 2.6.1. This transposon mutagenesis resulted in a UTI89 mutant library containing 320,000 mutants, equating to a coverage of approximately 1 insertion every 16 bp in UTI89, with a genome size of 5.18 Mb. The theoretical number of mutants required to generate a library of mutants with 99.99% coverage of a particular genome can be calculated by the following equation derived by (Zilsel et al., 1992):

$$n = \frac{\ln(1-P)}{\ln(1-f)}$$

Where n is the number of tranformants required, *P* is the probability that every transformant contains a transposon insertion and $f = \frac{transposon\ size\ (kb)}{genome\ size\ (kb)}$.

Using this equation it was determined that a library of 23,861 transformants would be required to obtain a library of Tn5 transposon (length 2.001 kb) mutants with a saturation of 99.99% in UTI89 (with a genome size of 5,179.971 kb). This indicated that the transposon library of 320,000 mutants generated for this thesis was likely sufficiently saturated. This is comparable to and on par with other published transposon sequencing studies which report a range of complexities. For example Eckert et al. (2011) used TraDIS to retrospectively study a library of 1,805 signature tagged mutants, whilst Subashchandrabose et al. (2013) studied the fitness of a library of 360,000 UPEC mutants in an *in vivo* model of systemic infection. In comparison, other studies have used much more saturated libraries. Langridge et al. (2009), for example, studied the essential genes for *Salmonella* Typhi using a library of 1.1 million transposon mutants whilst Phan et al. (2013)

generated a library of around 1 million *E. coli* ST131 mutants to study the genes required for serum resistance. Although these studies used transposon libraries with much higher insertion densities, the conditions used to screen these allow the screening of a larger number of mutants (1×10^8 CFU/ml in 500 ml (Eckert et al., 2011)). However, the microtitre plate assay needed to study the genes essential for biofilm formation at both 25°C and 37°C has a much lower inoculum of 2×10^6 CFU/200 µl (CFU/well) and is limited by the number of wells screened. Using this approach the library of 320,000 mutants was represented around 6.25 times in each well, and 30 wells were screened for each temperature (as described in Materials and Methods section 2.2.3).

5.4.2 Characterising population bottlenecks in biofilm formation

The microtitre plate assay was chosen for the TraDIS screening of the UTI89 transposon mutant library in order to identify potential genes involved in the type 1 pili independent biofilm formation phenotype previously identified in this thesis. The *in vitro* assay was chosen over the *in vivo* mouse model used previously as the latter has been shown to be subject to the founder effect, the loss of variation caused by a population bottleneck. Schwartz et al. (2011) characterised this population bottleneck in *in vivo* mouse infections whereby UPEC have to overcome several barriers in order to persist in the urinary tract. These barriers include urination, the mucosal surface and host immunity (Hannan et al., 2012). Only a small number of the inoculated bacteria are able to overcome these barriers and adhere to and invade the bladder epithelial cells, with the rest likely being flushed from the bladder during urination shortly after inoculation. Schwartz et al. (2011) showed that in the mouse model previously used in this thesis, of 10^7 CFU that are placed into the bladder at inoculation only 3-700 IBCs form by 6hpi, with each IBC being formed by one single founder cell. This population bottleneck would lead to a significant loss of diversity and would be particularly problematic with an extremely diverse transposon library.

It was hypothesised that the *in vitro* microtitre plate assay would present a less significant population bottleneck than the *in vivo* model. The UTI89 transposon mutant library generated for this thesis (previously described in section 5.4.1) contained approximately 320,000 different

mutations resulting in an estimated library complexity of around 1 insertion every 16bp. However, the complexity of the starting mutant library could be subject to several bottlenecks in the microtitre plate biofilm assay. The first possible bottleneck was the number of cells used for biofilm inoculation however, as described in section 5.4.1, this bottleneck wouldn't result in a loss of information as the bacterial input concentration was theoretically enough for all of the possible mutations to be represented in the TraDIS biofilm assay. A second major bottleneck may occur after inoculation and during the initial biofilm formation. If the founder effect was at play in the microtitre plate biofilm assay, i.e. if a single founder mutation was responsible for the entire biofilm studied at 24 hours, this would result in a major loss of diversity in the mutant library. In order to identify and characterise a possible population bottleneck during biofilm formation, a competitive biofilm formation experiment was carried out using varying ratios of WT UTI89 (SLC-6) and UTI89 with a kanamycin resistance marker (SLC-66) to seed biofilm formation (as described in Materials and Methods section 2.2.2). Several different mixtures of SLC66 and SLC-6 were created. Each inoculum mixture had a final concentration of 2×10^6 CFU/200 µl, but varying levels of kanamycin resistant cells (SLC-66) from 100% to 0.0001% CFU/200µl (in tenfold dilutions). The actual number of wildtype to kanamycin resistant cells present in each inoculum was confirmed by CFU enumeration (Materials and Methods section 2.4.6) and displayed in panel A of Figure 5.1.

After 24 hours of biofilm formation the planktonic cells were removed and titred (bacterial loads displayed in panel B of Figure 5.1) and the resulting biofilm was washed and then scraped with a cotton swab as detailed in Materials and Methods section 2.2.2. The swab heads were then placed in 1ml of LB media supplemented with 50µg/ml kanamycin and shaken at 37˚C for 4 hours in order to recover and detect any kanamycin resistant UTI89 cells. As shown in Figure 5.1 (panel B), WT UTI89 and kanamycin resistant UTI89 were present in the correct proportions in the inoculum strain mixtures with the kanamycin resistant cells being detected in the planktonic growth even when their starting proportions were as low at 0.0001% (2 CFU/well) suggesting that the outnumbered bacteria are able to reasonably proliferate in a cell mixture.

Finally, after 24 hours growth in the microtitre plate kanamycin resistant UTI89 cells were also recovered from the biofilm derived from all strain mixtures with starting proportions of 0.001% (20 CFU/well) and above (as shown in panel C of Figure 5.1). Although kanamycin resistant cells were not recovered after this 4 hour incubation from biofilms with a starting concentration of 0.0001% they were detected in the samples after 24 hours growth as depicted in Figure 5.2. This shows that cells in extremely low proportions (as little as 2 CFU/200 µl) are involved in biofilm formation and are not outcompeted by other cells in the environment. This suggests that there is not a population bottleneck in biofilm formation that will affect subsequent analysis when this complex library of mutants is used to seed biofilms in this microtitre plate biofilm assay.

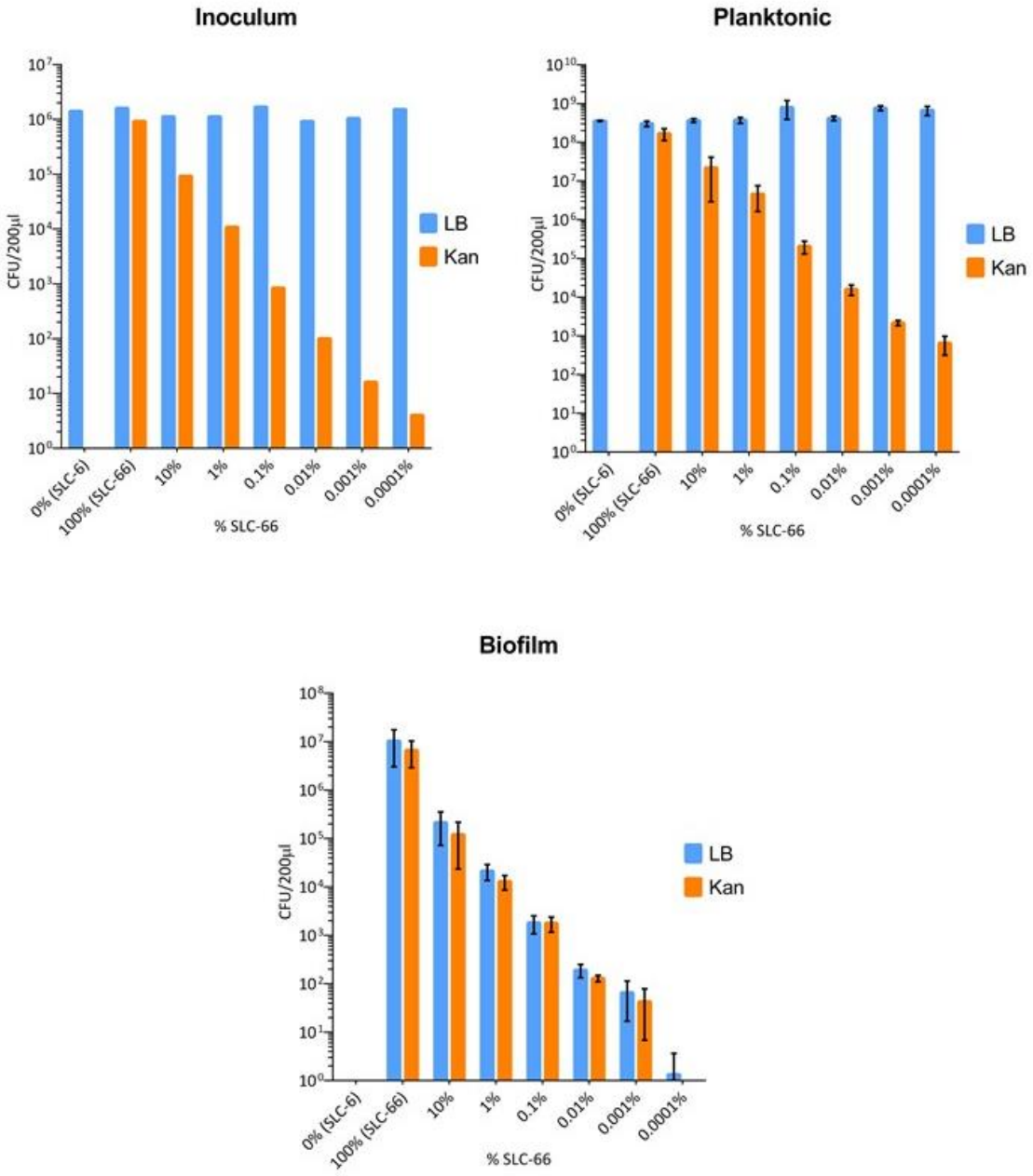


Figure 5.1 Characterising population bottlenecks in biofilms

Bacterial loads were calculated by CFU enumeration, plating on LB agar (blue bars) or LB agar supplemented with 50 µg/mL kanamycin (orange bars).

Panel A shows the bacterial load of strain mixtures used to seed biofilms in a competitive biofilm experiment in order to characterise potential bottlenecks. WT UTI89 (SLC-6) and kanamycin resistant UTI89 (SLC-66) were mixed in suspension to specific ratios with a total theoretical concentration of 2×10^6 CFU/ml.

Panel B shown the mean (n=3) bacterial load of planktonic cells per well (200 µl) after 24 hours biofilm growth at 37°C. Error bars represent ±1 standard deviation about the mean.

Panel C shown the mean (n=3) number of kanamycin resistant cells recovered from biofilms by swabbing and growth in LB/Kan for 4 hours, after 24 hours biofilm growth at 37°C. Error bars represent ±1 standard deviation about the mean.

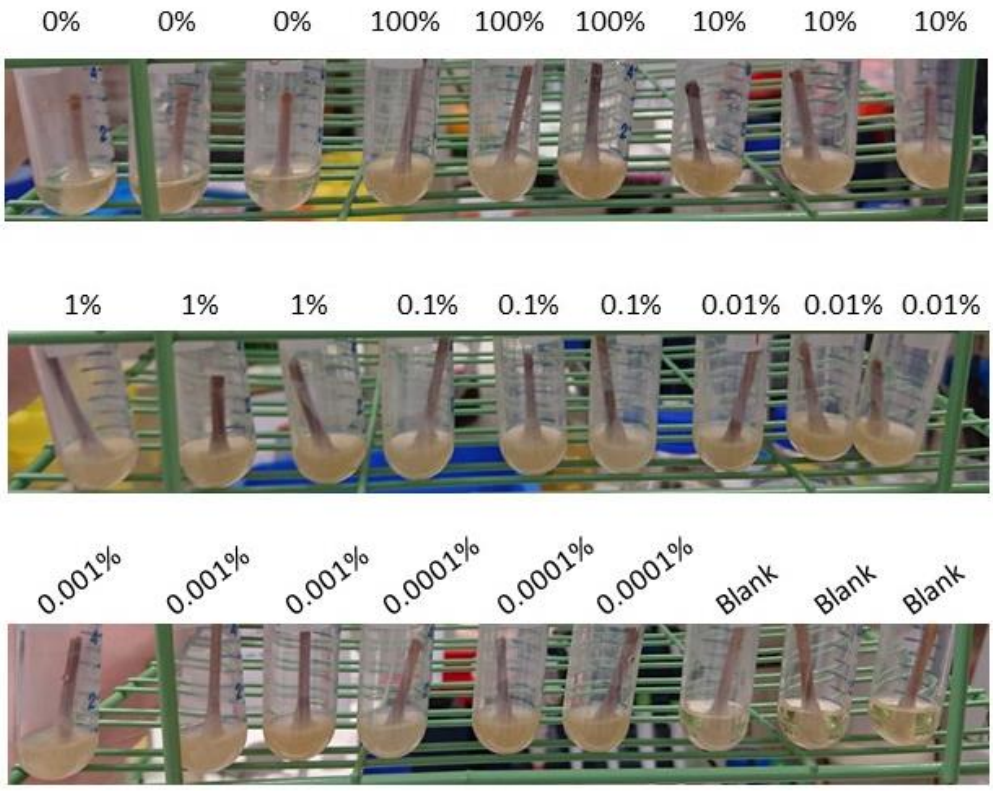


Figure 5.2 Recovered kanamycin resistant biofilm cells

Images showing the bacterial growth of the same samples as presented in Figure 5.1, but after being allowed to grow for a total of 24 hours in LB/kan. Growth of kanamycin resistant cells is evident in all SLC-66 dilutions, but not in the SLC-6 control (0% SLC-66) and the blank sterility controls.

5.4.3 Validation of UTI98 mutant library

As previously discussed in section 5.4.1, a library of transposon mutants was generated in WT UTI89. This mutant library consisted of 320,000 pooled transformants. In order to validate this library and determine the actual number of individual insertions present, TraDIS was performed on the library after 16 hours of growth (shaking in LB at 37°C) as described in Materials and Methods 2.6.2.2 and indicated in Figure 5.7). 42,205 individual insertions were detected by TraDIS in the mutant library (reported in Table 5.3). This accounts for 13.2% of the pooled library of 320,000 transformants and covers 0.8% all possible insertion positions in the UTI89 genome. These 42,205 individual insertions were in 2021 individual genes making up 37.7% of the total genes in *E.coli* strain UTI89. The positions of each individual insertion in the UTI89 genome and pUTI89 and the number of reads obtained for each are plotted in Figure 5.3 and Figure 5.4 respectively. A heavy insertion bias was present towards the origin of replication (Figure 5.3).

Standard validation of TraDIS experiments involves the calling of essential genes for growth and the comparison of this list to those in other published datasets. In this case, however, essential genes were not called due to the low coverage of insertions in the genome. The number of essential genes would be greatly overestimated due to the fact that 62.3% of genes did not contain a single insertion. However, two lists of essential genes were obtained from previously published datasets, those for EC5958 from a TraDIS study (Phan et al., 2013) and those from K-12 strain *E. coli* K-12 strain BW25113 (Baba et al., 2006a). The orthologs for each of these genes in UTI89 were established and the number of reads obtained for each gene by TraDIS in the mutant library sample were determined.

Baba et al. (2006a) generated a collection of 3985 single mutant strains in *E. coli* K-12 strain BW25113 by targeting 4288 individual genes, they were unable to disrupt 303 genes and list these as candidates for essential genes in this strain. Of the 303 genes deemed to be essential for BW25113, 298 orthologs were found in UTI89 and 262 of these (87.9%) contained no reads in the

UTI89 Tn5 mutant library. The remaining 36 genes contained between 1 and 204 reads each with the majority (25) having ≤5 reads. A total of 204 reads were obtained for *tnaB* and 100 for *bscB.* (Figure 5.5).

The list of essential genes for EC958 was from a TraDIS study performed by Phan et al. (2013). However, the reference genome used by these researchers is no longer publically available and the GenBank entry has been updated with a re-annotated version. This complicated the identification of orthologs for these genes in UTI89. 177 of the 315 genes determined to be essential by Phan et al. (2013) were successfully matched to the current version of the EC958 reference and orthologs for 143 of these genes were then found in UTI89. Of these 14 orthologs, only 6 (4.2%) were found to have reads in the UTI89 TraDIS mutant library and only 2 contained more than one read, EC958_3424 (or UTI89_C3454) with 14 reads and *psd* with 5 reads (Figure 5.6).

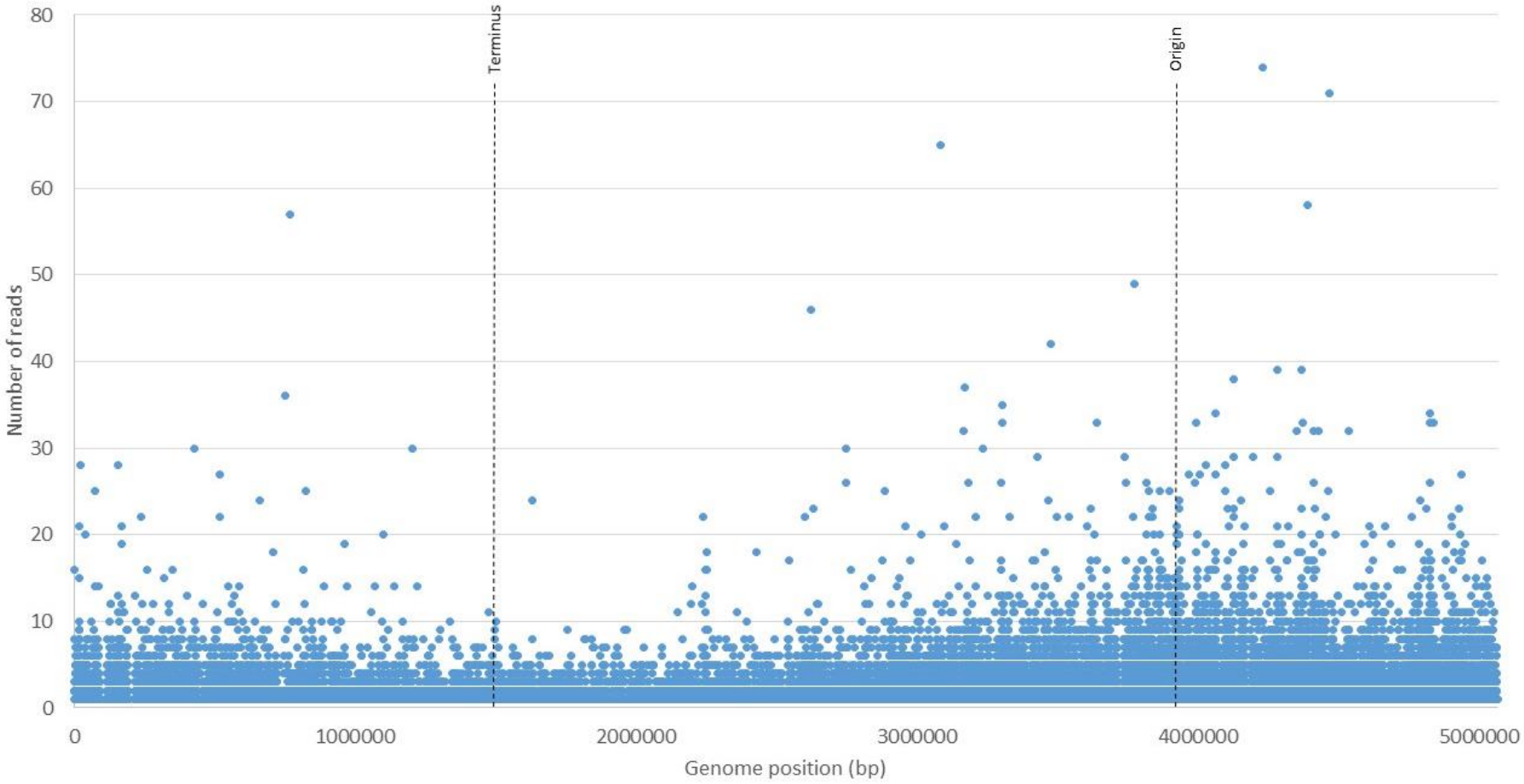


Figure 5.3 Location and number of reads for each individual insertion in the UTI89 Chromosome.

Scatter plot of the number of reads of obtained for each individual insertion. Each data point represents an individual insertion and the position of the origin and terminus of replication for UTI89 are marked

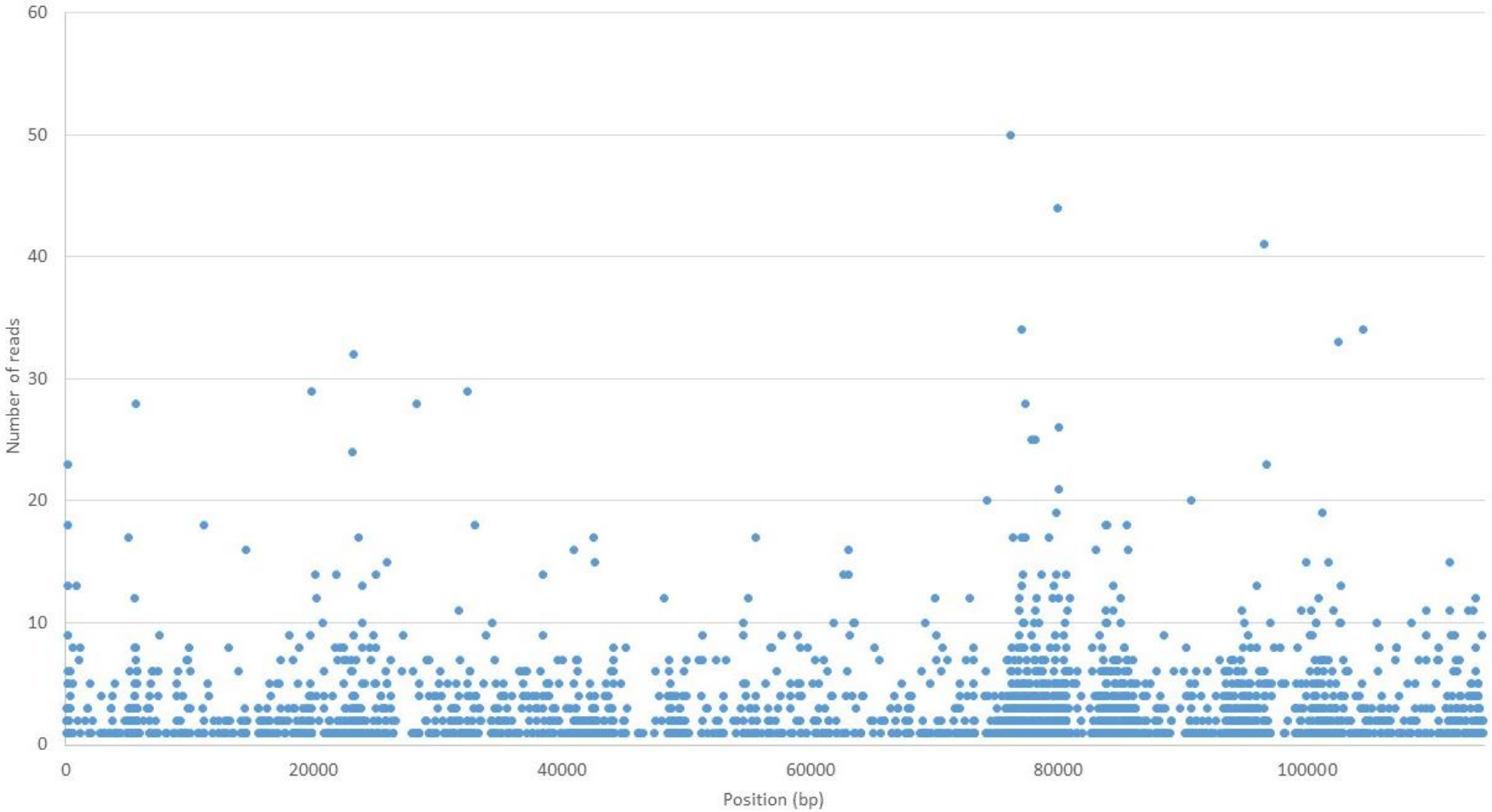


Figure 5.4 Location and number of reads for each individual insertion in the UTI89 plasmid (pUTI89).

Scatter plot indicating the number of reads for each individual insertion in the plasmid harboured by UTI89 (pUTI89).

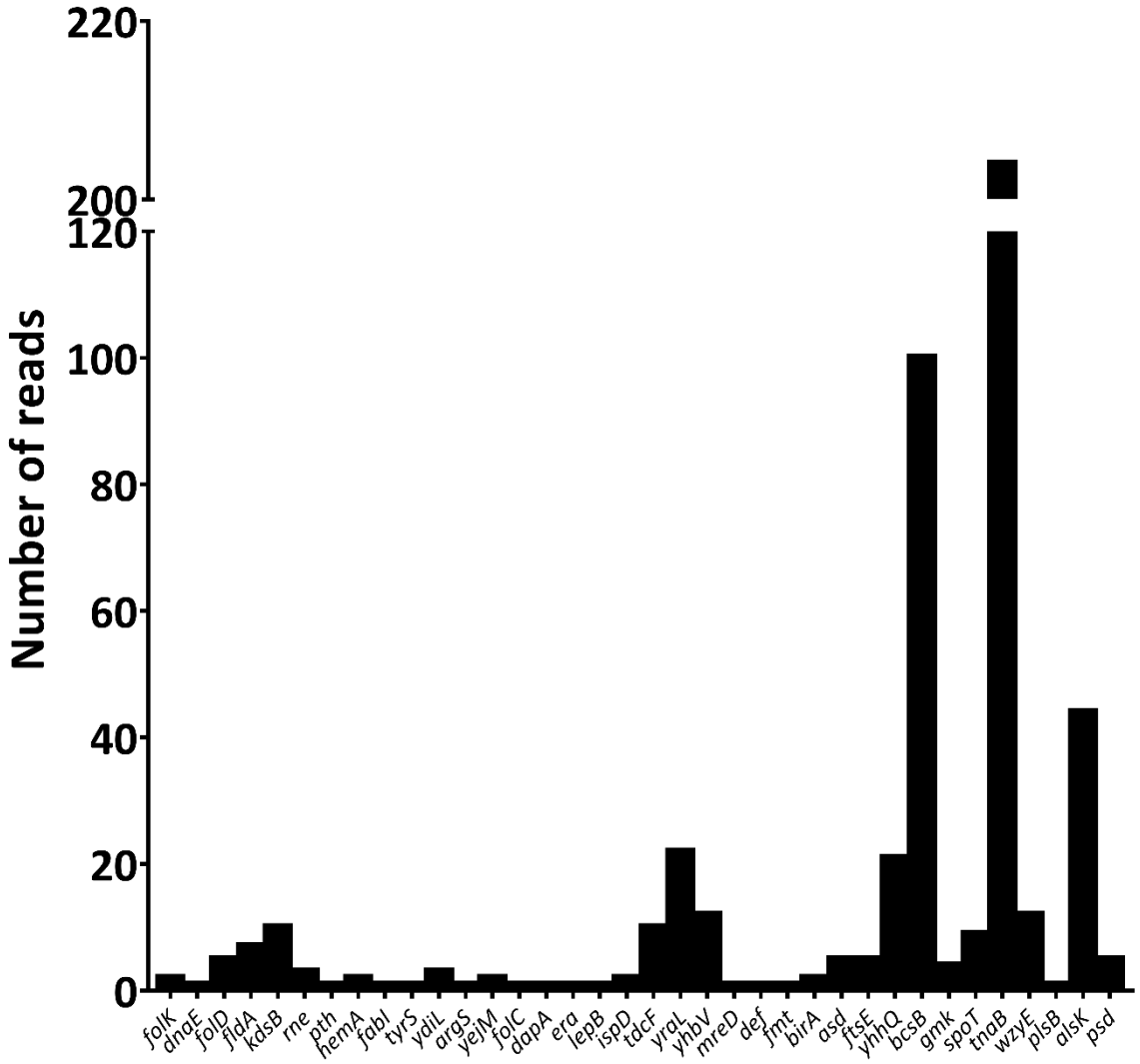


Figure 5.5 Reads obtained in Genes essential for growth in *E. coli* K-12 (BW25113)

Bar plot indicating the number of reads obtained for genes previously reported to be essential for growth in *E. coli* BW25113) in the mutant library. Only those genes that contained one or more reads are plotted here (for details of the number of reads obtained for previously reported essential genes in all TraDIS libraries see appendix B1)

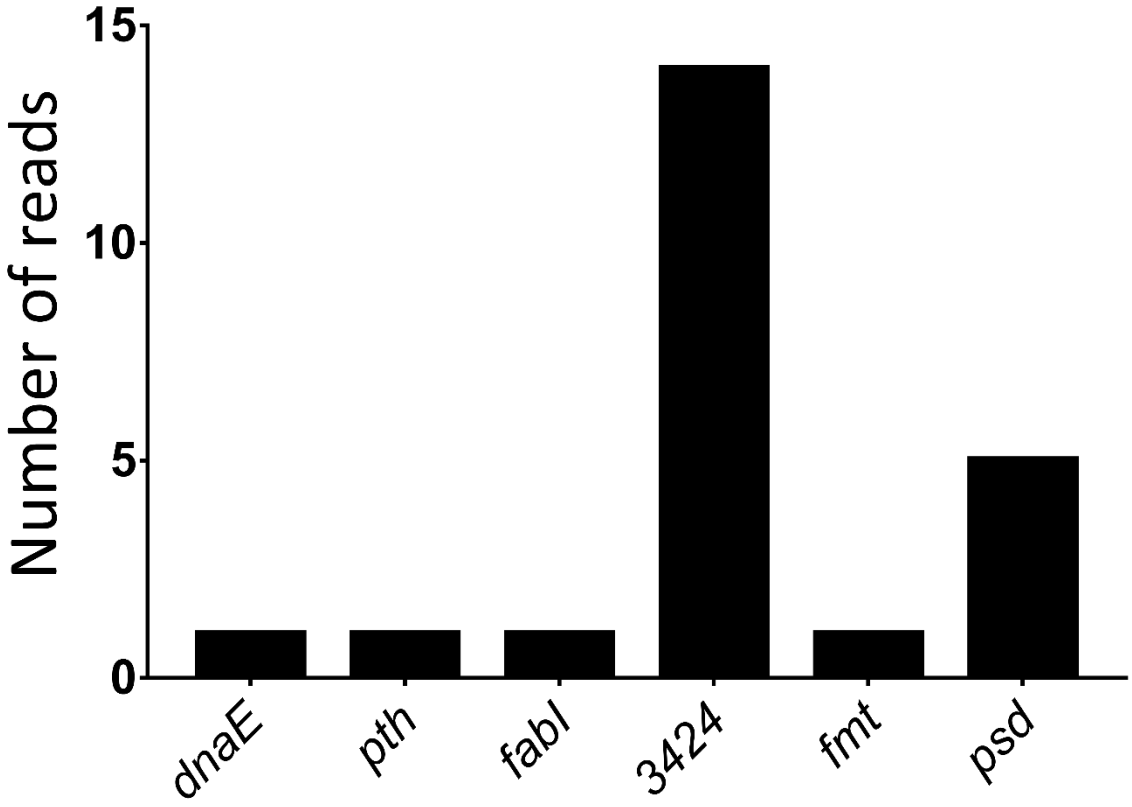


Figure 5.6 Reads obtained in genes previously found to be essential for growth in UPEC strain EC958

Bar plot indicating the number of reads obtained for genes previously reported to be essential for growth in *E. coli* EC958 in the mutant library. Only those genes that contained one or more reads are plotted here (for details of the number of reads obtained for previously reported essential genes in all TraDIS libraries see appendix B2)

5.4.4 TraDIS to identify biofilm factors

Transposon directed insertion site sequencing was carried out using the UTI89 transposon mutant library described in Section 5.4.1. The mutant library was passaged in biofilm specific conditions and the DNA from the resulting cells was extracted and the transposon insertion junctions sequenced (as described in Materials and Methods section 2.6) in order to determine genes essential for said conditions. The conditions tested were biofilm formation at 25°C and 37°C, both the biofilm and the planktonic fractions were recovered for sequencing. The input mutant library was also sequenced along with two static growth (in LB) controls, one at 25°C and one at 37°C as described in Figure 5.7.

CFU enumeration was performed for each of the samples to be sequenced prior to DNA extraction (excluding the biofilm fraction as DNA was extracted directly from cotton swabs for these samples). Aliquots were serially diluted and plated on both LB and LB/kan in order to confirm the cells present contained the transposon and that the input concentrations were correct. Bacterial titres are displayed in Table 5.1. Bacterial titres are comparable on both LB and LB/kan indicating that all cells present contained a transposon and therefore kanamycin resistant. The concentration of the input cells was as expected, in the region of 1×10^6 CFU/ml, and can be seen to have proliferated to around 1×10^9 in the biofilm assay and the static controls, as is reflected in the bacterial concentration of the planktonic fraction and the static control shown in Table 5.1.

Table 5.1 CFU enumeration for TraDIS samples

Aliquots of each TraDIS sample were taken prior to DNA extraction to confirm the bacterial load. Cells were subject to CFU enumeration and titres were plated onto both LB and LB/kan. The mutant library was grown up overnight in LB at 37°C before being titred. The concentration was then normalised and diluted to become the screening input population, which was titred to confirm the input concentration for both biofilm assays and static controls. CFU enumeration of the planktonic fractions and static controls were performed after static growth at either 25°C or 37°C.

Repeat 1

Sample	LB (CFU/ml)	Kan (CFU/ml)
Mutant library	5.4×10^{7}	6.2×10^{7}
Screening input	1.1×10^{6}	1.28×10^{6}
25°C static	7.6×10^{9}	7.40×10^{9}
25°C planktonic	6.8×10^{8}	5.8×10^{8}
37°C static	4.4×10^{9}	1.68×10^{9}
37°C planktonic	4.8×10^{9}	6.00×10^{9}

Repeat 2

Sample	LB (CFU/ml)	Kan (CFU/ml)
Mutant library	1.28×10^{9}	1.06×10^{9}
Screening input	1×10^{6}	1.36×10^{6}
25°C static	5.6×10^{9}	5.00×10^{9}
25°C planktonic	5.2×10^{8}	6×10^{8}
37°C static	6.8×10^{8}	9.2×10^{8}
37°C planktonic	1.62×10^{9}	4.4×10^{9}

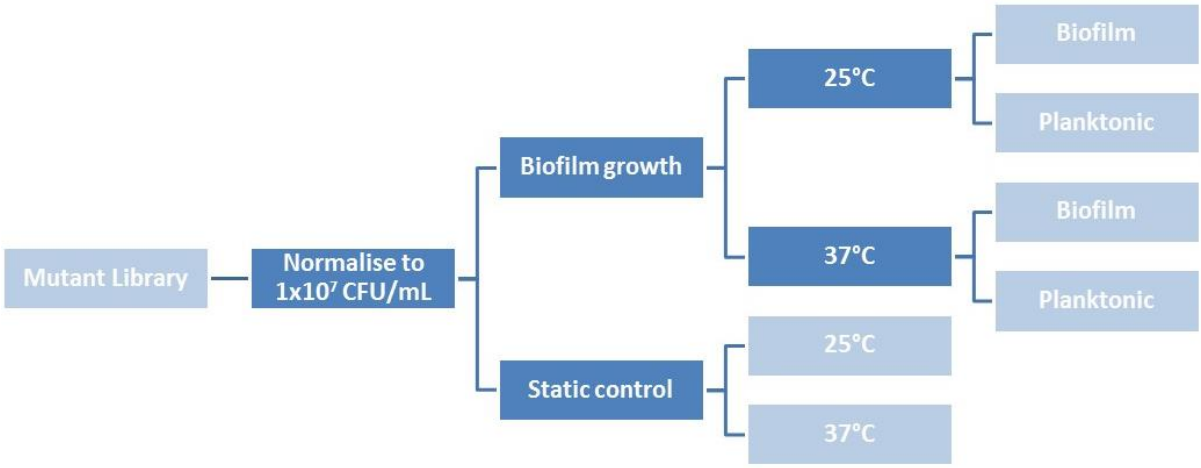
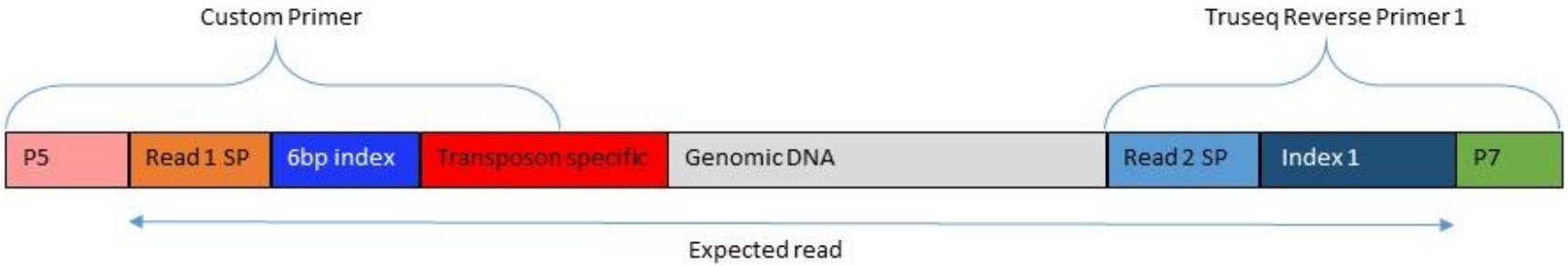


Figure 5.7 Experimental design flowchart of TraDIS library screening.

Flowchart summarising the experimental process utilised to screen the transposon mutant library under a range of different conditions as described in Materials and Methods section 2.6. The mutant library was grown up in 50 ml LB overnight before normalised aliquots were screened in several test conditions. Light blue boxes denote those samples sequenced by TraDIS.

TraDIS libraries were sequenced using the Illumina HiSeq 4000 system. The TraDIS sequencing data obtained was de-multiplexed according to the 6 bp index (included by the custom library amplification primer during sequencing library preparation (Table 2.3)) and the transposon specific sequence indicated in Figure 2.1, and mapped to the UTI89 reference genome (Chen et al., 2006), containing both the chromosomal sequence and that of pUTI89 (see Materials and Methods section 2.6.4.5). One sequencing run was performed and a total of 38,114,270 mapped reads were obtained from a single lane. This is substantially lower than the expected output of the HiSeq 4000 system which should be around 500-600 million reads per lane. (Illumina, 2015). This was due to a lack of diversity in the first few bases of each sequencing read, and was a result of every read containing the same transposon specific sequence. The base percentage for the first 25 cycles of each sequencing run should be balanced (each base represented in equal proportions) for efficient and optimal cluster calling (Illumina, 2016).

High levels of mis-priming were also observed after demultiplexing, due to random priming across the genome by the transposon specific library amplification primer. In order to overcome this, sequencing data was subject to a second de-multiplexing step using the end of the transposon sequence present in the reads (see Figure 2.1). Although this second round of filtering provided a more accurate set of transposon specific reads it also substantially reduced the number of reads present for further analysis with between only 2% and 15% of mapped reads being on target (Table 5.2). The number of mapped reads in each library was determined using SAMtools (Li et al., 2009)



Expected amplicon sequence:

5'-AATGATACGGCGACCACCGAGATCT ACACTCTTTCCCTACACGACGCTCTTCCGATCT **XXXXXX** ACCTACAACAAAGCTCTCATCAACC END-OF-TRANSPOSON GENOMIC-DNA GAACTCCAGTCAC **ATCACG** ATCTCGTATGCCGTCTTCTGCTTG**-3'**

Expected read sequence:

5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCT **XXXXXX** ACCTACAACAAAGCTCTCATCAACC END-OF-TRANSPOSON GENOMIC-DNA GAACTCCAGTCAC **ATCACG-3'**

Figure 5.8 TraDIS rationale - expected amplicon and read structure

Schematic diagram to outline the structure of expected amplicons for TraDIS sequencing and the resulting sequencing reads. Primer sequences are detailed in Table 2.3.

Table 5.2 Summary of read numbers from each of the TraDIS sequencing libraries.

The number of mapped reads for each library in the sequencing run after each round of de-multiplexing and the percentage on target after the second round. Percentage on target is the proportion of DEMUX 1 mapped reads remaining after DEMUX 2. Samples annotated by a hyphen (-) are other TraDIS libraries generated with the same transposon but were not sequenced for this thesis; no further analysis was performed with these samples for this thesis however read numbers are shown here to describe sequencing statistics for the entire run. Two biological replicates, (1) and (2), were sequenced on the same run.

Index (AD)	Sample (replicate)	Number of Mapped reads DEMUX 1	Number of Mapped reads DEMUX 2	% on target
AD001	Mutant library (1)	282,966	7,828	2.8
AD002	-	428,852	25,497	5.9
AD003	-	563,413	57,940	10.3
AD004	-	3,936,541	177,187	4.5
AD005	-	1,018,999	43,711	4.3
AD006	37°C biofilm (1)	371,595	11,411	3.1
AD007	37°C planktonic (1)	3,012,481	121,198	4.0
AD008	37°C static (1)	733,072	19,768	2.7
AD009	25°C biofilm (1)	275,001	27,057	9.8
AD010	25°C planktonic (1)	615,551	32,957	5.4
AD011	25°C static (1)	1,000,380	48,247	4.8
AD012	-	2,323,760	104,378	4.5
AD013	Mutant library (2)	2,451,799	96,182	3.9
AD014	-	240,370	36,933	15.4
AD015	-	1,644,630	83,159	5.1
AD016	-	13,115,074	438,431	3.3
AD018	-	913,815	49,398	5.4
AD019	37°C biofilm (2)	664,005	31,913	4.8
AD020	37°C planktonic (2)	1,316,819	55,490	4.2
AD021	37°C static (2)	279,993	14,211	5.1
AD022	25°C biofilm (2)	345,377	14,318	4.1
AD023	25°C planktonic (2)	1,063,522	75,010	7.1
AD025	25°C static (2)	1,065,834	60,307	5.7
AD027	-	450,421	11,816	2.6
	Total	**38,114,270**	**1,644,347**	**4.3**

Table 5.3 Total number of reads for each TraDIS screening condition

The total number of reads from each TraDIS screening condition tested in this thesis (after 2 rounds of demultiplexing and the number of individual insertions observed in each library.. Reads were pooled from two biological replicates sequenced on one sequencing run.

TraDIS sample	Total number of reads	Total number of individual insertions
Mutant library	104,010	42,205
37°C biofilm	43,324	24,049
37°C planktonic	176,688	49,487
37°C static	33,979	20,923
25°C biofilm	41,375	23,765
25°C planktonic	107,967	42,410
25°C static	108,554	43,310

The number of reads present in each gene was determined. As can be seen in Figure 5.9 and Figure 5.10 each sample has a very similar distribution of insertions in both the chromosome and pUTI89 (the plasmid harboured by UTI89). These distributions also suggest the possibility of a substantial insertion bias, with all samples having larger numbers of insertions in the same regions. The data in these figures however show the raw counts per gene and is therefore skewed by the length of each gene – longer genes are likely to have more insertions. In order to overcome this an insertion index was calculated for each gene by dividing the number of reads per gene by the gene length (bp) as described by Langridge et al. (2009). The equation used to determine an insertion index for each gene was as follows:

$$Insertion\ Index = \frac{Number\ of\ reads}{Gene\ length\ (bp)}$$

The number of insertions per gene normalised by gene length for each sample are displayed in Figure 5.11 (UTI89 Chromosome) and Figure 5.12 (pUTI89). This normalisation however did not correct for the large numbers of insertions in specific regions and there is still some evidence of a potential insertion bias that is not a result of gene length. However, the insertion indexes are similar for each gene between samples, with all samples displaying a similar read distribution.

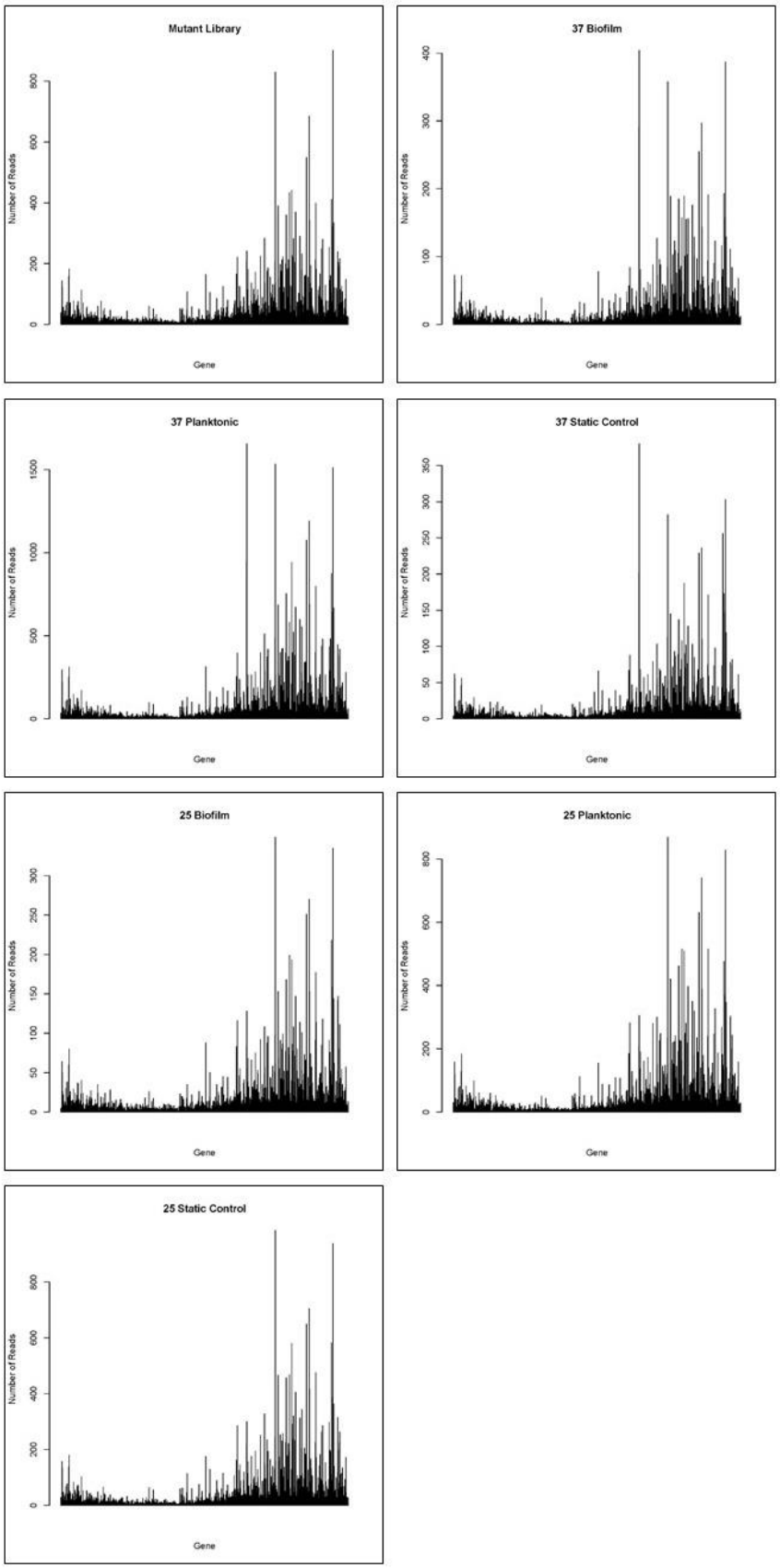


Figure 5.9 Chromosomal insertion distribution of TraDIS samples

Bar graph showing the number of reads obtained for each gene in the UTI89 chromosome for each TraDIS sample.

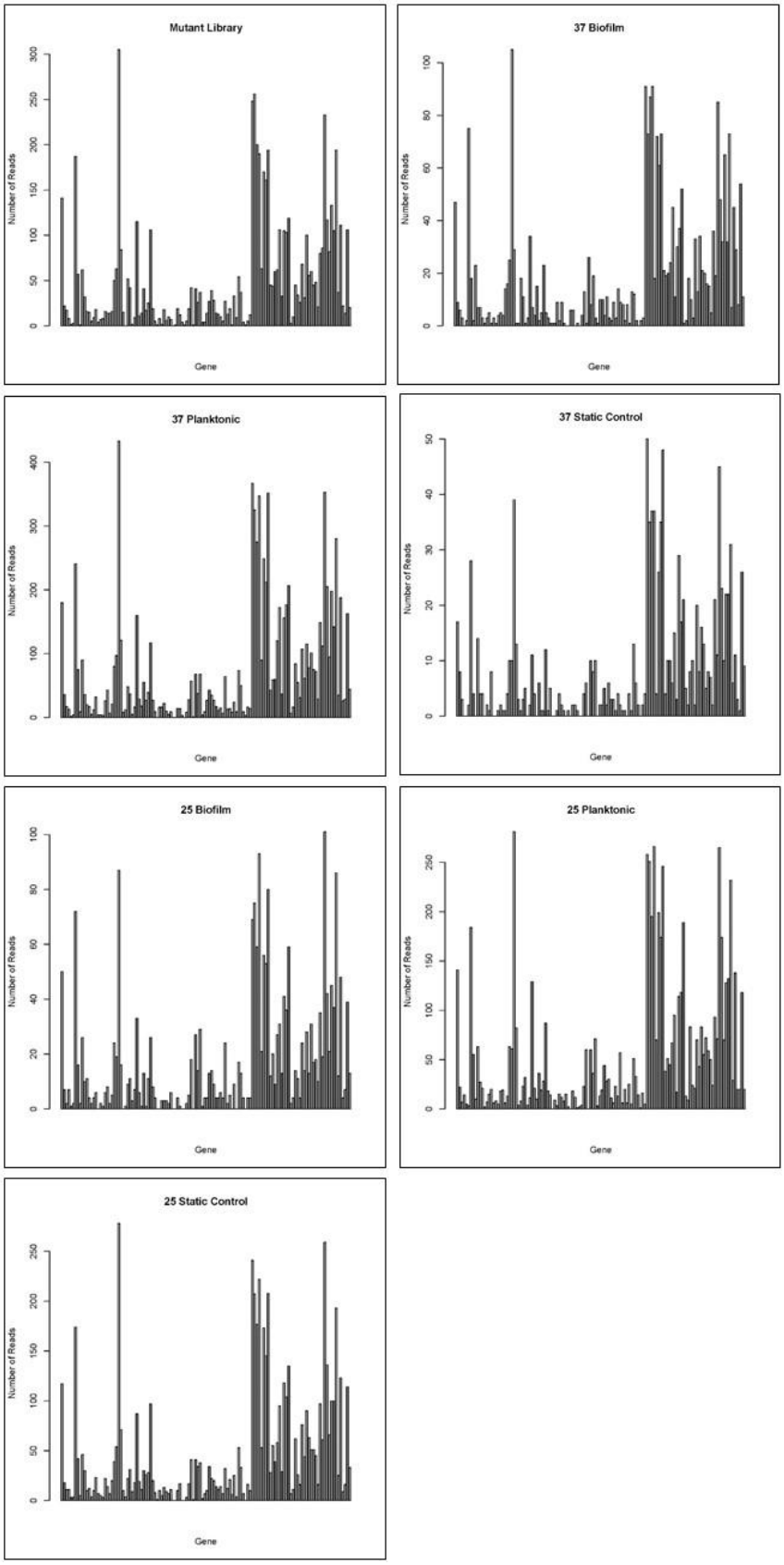


Figure 5.10 pUTI89 insertion distribution of TraDIS samples

Bar graph showing the number of reads obtained for each gene in the plasmid harboured by UTI89 (pUTI89) for each TraDIS sample.

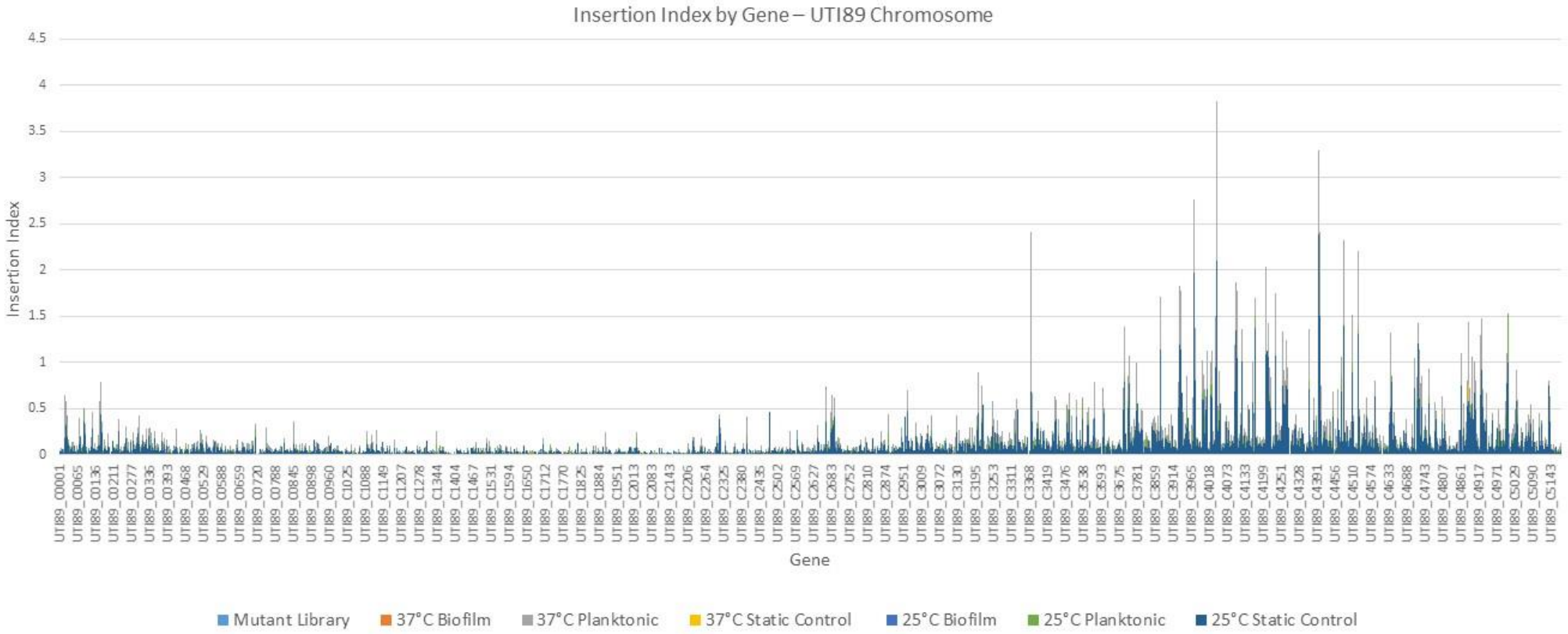


Figure 5.11 UTI89 Chromosomal insertion distribution normalised by read length

The number of reads obtained for each gene of the UTI89 chromosome was normalised by the length of the respective gene to create an insertion index ($Insertion\ Index = \frac{Number\ of\ reads}{Gene\ length\ (bp)}$). Overlayed bar graphs show the insertion index for each gene in the UTI89 chromosome for each of the TraDIS samples.

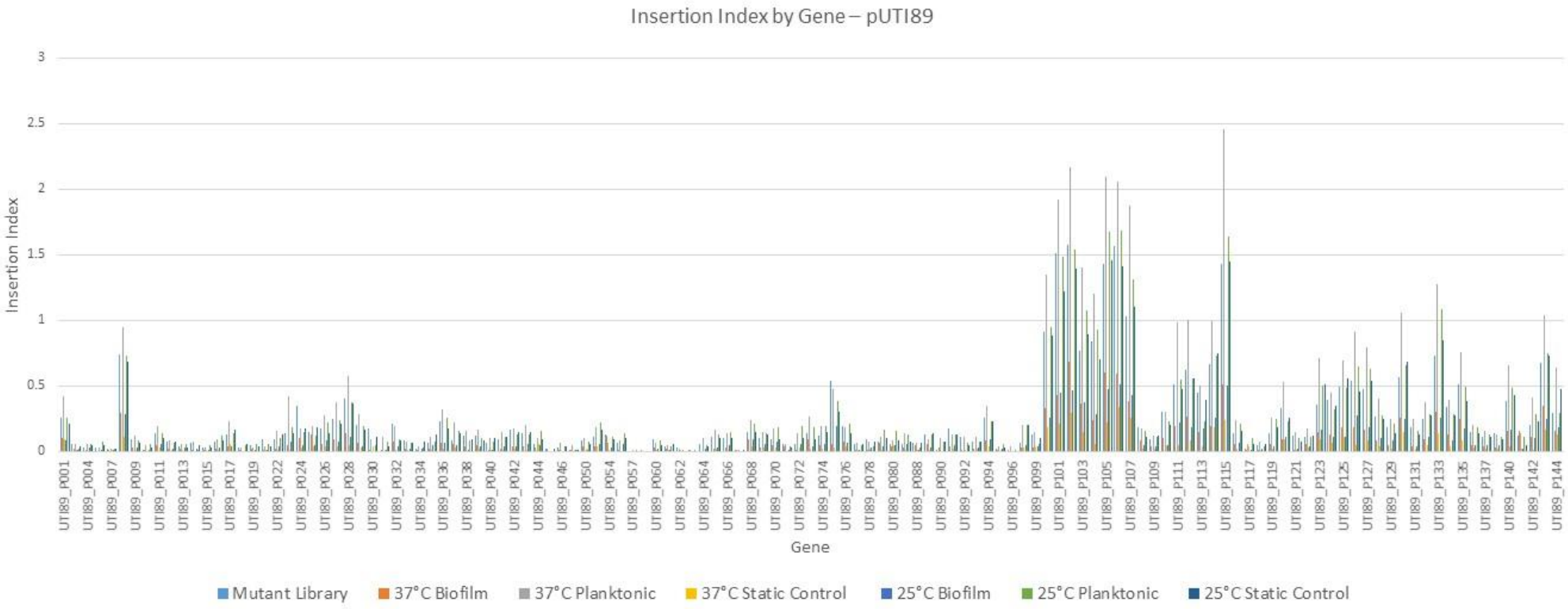


Figure 5.12 pUTI89 insertion distribution normalised by gene length

The number of reads obtained for each gene of pUTI89 was normalised by the length of the respective gene to create an insertion index ($Insertion\ Index = \frac{Number\ of\ reads}{Gene\ length\ (bp)}$). Overlayed bar graphs show the insertion index for each gene in the UTI89 chromosome for each of the TraDIS samples.

Pairwise comparisons were carried out between samples in order to identify differences in read numbers for each gene. For this purpose, a proportion test was carried out in R (R Development Core Team, 2013) using the prop.test function in order to determine the likelihood by which the number of reads for a given gene differ between two libraries. Quantile-Quantile (Q-Q) plots were generated (as described in Materials and Methods section 0) in order to visualise the data generated from this test statistic and aid in the identification of genes that were most likely to differ in the number of reads, between two different libraries (Figure 5.13). These Q-Q plots compare the observed *p* values against a uniform distribution to identify values (genes) that differ from the norm.

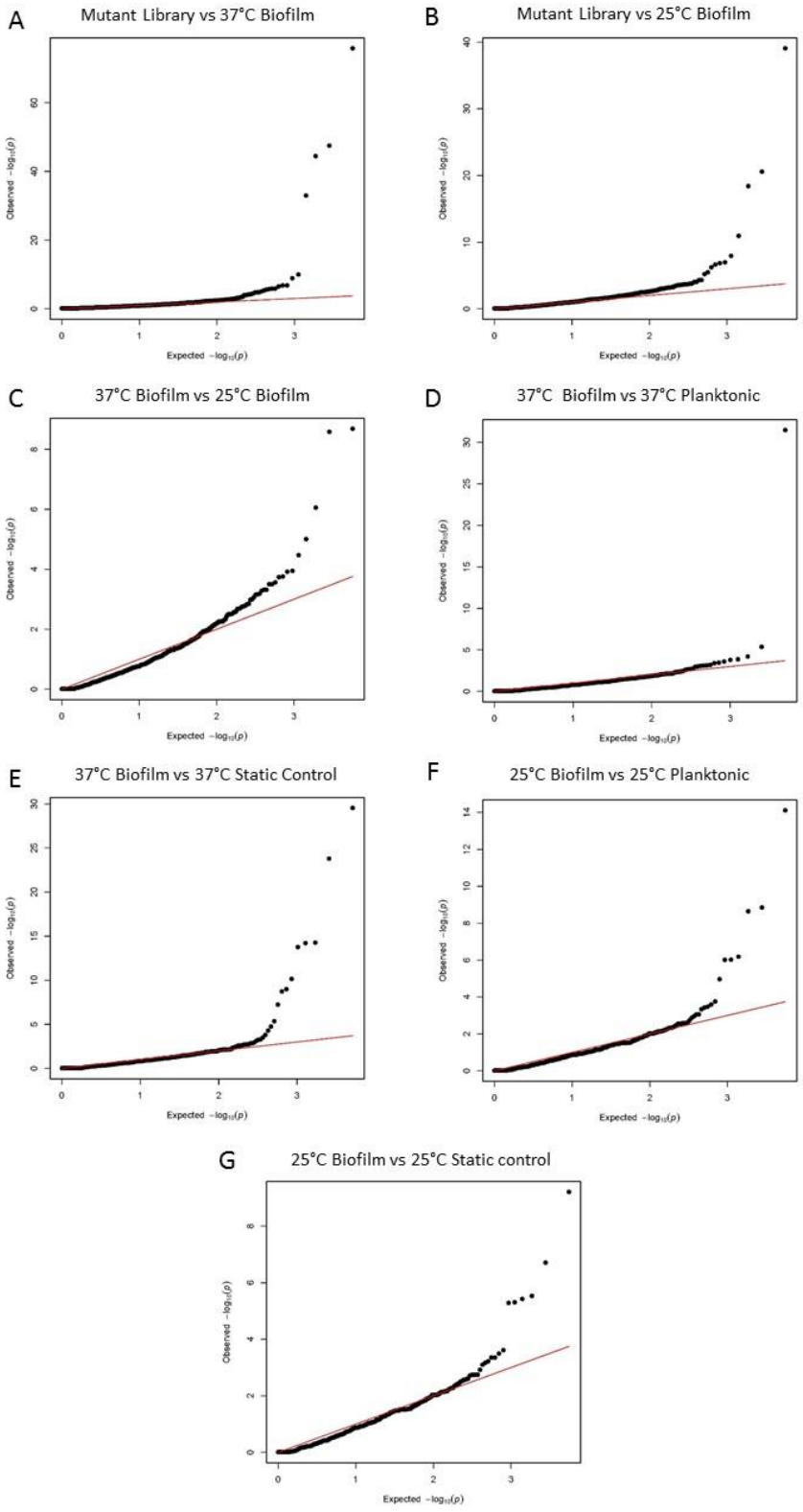


Figure 5.13 Q-Q plots of significance values for proportion test comparisons of TraDIS data.

Q-Q plots were generated in R to visualise the genes with P values that differed from a normal distribution in pairwise comparisons.

The significance values were then corrected using the Benjamini-Hoshberg approach (Benjamini and Hochberg, 1995), in order to control for type 1 error (false positives), this was again performed in R (Base package, version 3.0.0) and the corrected values were used for subsequent analysis. The most significant of the corrected p-values for each comparison and the genes to which they corresponded were then identified. The genes of most significance for each comparison are detailed in Table 5.4, Table 5.5, Table 5.6, Table 5.7, Table 5.8, Table 5.9 and Table 5.10. Several genes potentially involved in biofilm formation at 37°C can be identified from these significant differences in read/insertion numbers identified by pairwise comparisons between test conditions. These genes include *rbsR, rbsK, yjcC, rfaJ, rfaq, ybhJ, yhiX, waaL* and *waaV.* When comparing the number of reads for each gene in the 25°C and 37°C biofilm samples several genes, *rbsR, rbsK , fim E* and *yjcC,* were seen to have significantly less reads in the 37°C biofilm sample compared to that at 25°C (Table 5.6) suggesting they may play a role in biofilm formation at 37°C. . Alongside these, several other genes (*yhiX*, *rfaJ*, *rfaQ*, *ybhJ*, *waaV* and *waaL*) had significantly less reads in the 37°C biofilm compared to the input library (Table 5.4), again suggesting a potential role for these in biofilm formation at 37°C.

It is interesting to note that several of these genes are members of the same operons (determined using EcoCyc (Keseler et al., 2013)). For example, *rfaJ, rfaQ, waaL* and *waaV are* all members of the major core –oligosaccharide operon in *E. coli.* As well as these, the *rbsR* and *rbsK* genes are both members of the same operon and are involved in ribose degradation. Also of particular interest was that 0 reads were detected in *fimD* in the 25°C biofilm sample and this was significantly fewer than the 204 detected in the mutant library and the 41 in the 37°C biofilm. The *fimD* gene encodes the usher protein responsible for surface localisation of type 1 pili and this result therefore confirms previous data that type 1 pili are essential for *in vitro* biofilm formation at 25°C. Several genes in the K1 capsule gene cluster, *neuD, neuC, neuB* and *kpsM,* had significantly more reads in the 37°C biofilm than the 25°C biofilm (Table 5.6) suggesting that they are not required for biofilm formation at 37°C.

Table 5.4 Mutant library vs 37°C biofilm read number comparison

A proportion test was carried out in R in order to compare the number of reads for each gene and determine the probability that these proportion differ. All genes with significant difference between the number of reads present in the mutant library input and 37°C biofilm are displayed in this table. Where two genes are displayed separated by a by a forward slash (/) this represents an intergenic insertion between the two named genes. The total number of all mapped reads for each library is displayed in the bottom row for reference.

Gene	Product	Location	Mutant library (reads per gene)	37°C Biofilm (reads per gene)	P value
neuD	Sialic acid synthase NeuD	Chromosome	241	404	7.88E-73
neuB	Sialic acid synthase NeuB	Chromosome	148	250	8.67E-45
neuC	Polysialic acid biosynthesis protein P7	Chromosome	209	291	6.43E-42
kpsM/gspM	Polysialic acid transport protein KpsM/ putative general secretion pathway protein YghD	Chromosome	82	155	1.45E-30
flhD/yecG	Transcriptional activator FlhD/universal stress protein UspC	Chromosome	5	26	1.17E-07
yhiX	DNA binding transcriptional regulator GadX	Chromosome	196	23	1.19E-06
yicE	Putative transport protein	Chromosome	25	38	1.13E-04
oxyR	DNA-binding transcriptional regulator OxyR	Chromosome	19	33	1.13E-04
C2915/C2924	Putative transposase InsK for insertion sequence element IS150/hypothetical protein	Chromosome	118	11	2.00E-04
ccmC	Ccme heme exported protein C	Chromosome	2	14	7.06E-04
***murb/*C3813**	UDP-N-acetylenolpyruvoylglucosamine reductase/hypothetical protein	Chromosome	98	8	7.06E-04
yieE	Hypothetical protein	Chromosome	3	15	8.85E-04
typA	GTP binding protein	Chromosome	62	60	1.14E-03
kpsT	Polysialic acid transport ATP-binding protein KpsT	Chromosome	35	41	1.94E-03
rfaJ	Lipopolysaccharide 1,2-glucosyltransferase	Chromosome	128	18	3.35E-03

Table 5.5 continued.

Gene	Product	Location	Mutant library (reads per gene)	37°C Biofilm (reads per gene)	P value
mprA	Transcriptional repressor MprA	Chromosome	2	12	5.17E-03
rfaQ	Lipopolysaccharide core biosynthesis protein	Chromosome	68	4	5.30E-03
fimD	Outer membrane usher protein FimD precursor	Chromosome	204	41	5.65E-03
finO	Conjugal transfer fertility inhibition protein FinO	Plasmid	22	29	9.74E-03
ybhJ	Hypothetical protein	Chromosome	76	7	1.29E-02
lrhA	Transcriptional regulator LrhA	Chromosome	6	15	1.70E-02
ydhZ/yrdA	Amino acid ABC transporter ATP-binding protein/ protein YrdA	Chromosome	124	20	1.70E-02
C4367	Hypothetical protein	Chromosome	290	176	2.17E-02
waaV	Putative beta1,3-glucosyltransferase	Chromosome	149	28	2.27E-02
waaL	Putative lipid A-core surface polymer ligase	Chromosome	68	6	2.27E-02
		Total reads	104,010	93,109	

Table 5.5 Mutant library vs 25°C biofilm read number comparison

A proportion test was carried out in R in order to compare the number of reads for each gene and determine the probability that these proportion differ. All genes with significant difference between the number of reads present in the mutant library input and 25°C biofilm are displayed in this table. Where two genes are displayed separated by a forward slash (/) this represents an intergenic insertion between the two named genes. The total number of all mapped reads for each library is displayed in the bottom row for reference.

Gene	Function	Location	Mutant library (reads per gene)	25°C Biofilm (reads per gene)	P value
fimE	Tyrosine recombinase	Chromosome	64	147	4.97E-36
rbsR	Transcriptional repressor RbsR	Chromosome	37	80	7.87E-18
fimD	Outer membrane usher protein FimD precursor	Chromosome	204	0	7.52E-16
rbsK	Ribokinase	Chromosome	31	52	1.65E-08
yfiR	Hypothetical protein	Chromosome	16	32	1.30E-05
yciR	Rnase II stability modulator	Chromosome	4	18	1.02E-04
lrhA	Transcriptional regulator LrhA	Chromosome	6	20	1.16E-04
ydiV/nlpC	Hypothetical protein/lipoprotein NlpC	Chromosome	0	12	1.63E-04
yidR	Hypothetical protein	Chromosome	29	38	3.75E-04
yhiX	DNA-binding transcriptional regulator GadX	Chromosome	196	33	1.95E-03
wzzE	Lipopolysaccharide biosynthesis protein WzzE	Chromosome	103	10	3.22E-03
C3251	Xanthine dehydrogenase subunit XdhA	Chromosome	96	11	2.17E-02
neuA	Acylneuraminate cytidylyltransferase	Chromosome	171	31	2.17E-02
yddB	Hypothetical protein	Chromosome	7	15	3.72E-02
waaV	Putative beta1,3-glucosyltransferase	Chromosome	149	26	3.72E-02
		Total reads	104,010	41,375	

Table 5.6 37°C Biofilm vs 25°C biofilm read number comparison

A proportion test was carried out in R in order to compare the number of reads for each gene and determine the probability that these proportion differ. All genes with significant difference between the number of reads present in the 37°C biofilm and the 25°C biofilm are displayed in this table. Where two genes are displayed separated by a forward slash (/) this represents an intergenic insertion between the two named genes. The total number of all mapped reads for each library is displayed in the bottom row for reference.

Gene	Function	location	37°C Biofilm (reads per gene)	25°C Biofilm (reads per gene)	P value
neuD	Sialic acid synthase NeuD	Chromosome	404	128	1.50E-26
neuC	Polysialic acid biosynthesis protein P7	Chromosome	291	84	4.21E-21
neuB	Sialic acid synthase NeuB	Chromosome	250	96	7.88E-12
fimE	Tyrosine recombinase	Chromosome	43	147	7.88E-12
kpsM/gspM	Polysialic acid transport protein KpsM/putative general secretion pathway protein YghD	Chromosome	155	42	1.79E-11
typA	GTP-binding protein	Chromosome	60	5	6.12E-08
fimD	Outer membrane usher protein FimD precursor	Chromosome	41	0	7.59E-07
rsbR	Transcriptional repressor RbsR	Chromosome	21	80	1.20E-06
neuA	Acylneuraminate cytidylyltransferase	Chromosome	96	31	3.29E-05
yjcC	Hypothetical protein	Chromosome	57	114	2.27E-03
rbsK	Ribokinase	Chromosome	17	52	8.41E-03
finO	Conjugal transfer fertility inhibition protein FinO	Plasmid	29	4	2.21E-02
		Total reads	43,324	41,375	

Table 5.7 37°C biofilm 37 vs 37°C planktonic read number comparison

A proportion test was carried out in R in order to compare the number of reads for each gene and determine the probability that these proportion differ. All genes with significant difference between the number of reads present in the 37°C biofilm and 37°C planktonic samples are displayed in this table. Where two genes are displayed separated by a forward slash (/) this represents an intergenic insertion between the two named genes. The total number of mapped reads for each library is displayed in the bottom row.

Gene	Function	location	37°C Biofilm (reads per gene)	37°C Planktonic (reads per gene)	P value
kpsT	Polysialic acid transport ATP-binding protein KpsT	Chromosome	41	50	7.51E-06
finO	Conjugal transfer fertility inhibition protein FinO	Plasmid	29	26	7.51E-06
ccmC	Heme exporter protein C	Chromosome	14	8	1.69E-03
kpsM	Polysialic acid transport protein KpsM	Chromosome	78	174	1.42E-02
hybE	Hydrogenase 2-specific chaperone	Chromosome	22	28	3.90E-02
		Total reads	43,324	176,688	

Table 5.8 37°C biofilm vs 37°C static control read number comparison

A proportion test was carried out in R in order to compare the number of reads for each gene and determine the probability that these proportion differ. All genes with significant difference between the number of reads present in the 37°C biofilm and the 37°C static control are displayed in this table. Where two genes are displayed separated by a forward slash (/) this represents an intergenic insertion between the two named genes. The total number of mapped reads for each library is displayed in the bottom row.

Gene	Function	Location	37°C Biofilm (reads per gene)	37°C static control (reads per gene)	P value
papG	P pilus adhesin PapG protein	Chromosome	81	256	1.73E-28
kpsT	Polysialic acid transport ATP-binding protein KpsT	Chromosome	41	4	1.10E-02
		Total reads	43,324	33,979	

Table 5.9 25°C biofilm vs 25°C planktonic read number comparison

A proportion test was carried out in R in order to compare the number of reads for each gene and determine the probability that these proportion differ. All genes with significant difference between the number of reads present in the 25°C biofilm and the 25°C planktonic samples are displayed in this table. Where two genes are displayed separated by a forward slash (/) this represents an intergenic insertion between the two named genes. The total number of mapped reads for each library is displayed in the bottom row.

Gene	Function	Location	25°C Biofilm (reads per gene)	25°C Planktonic (reads per gene)	P value
rbsR	Transcriptional repressor RbsR	Chromosome	80	59	4.28E-11
yjcC	Hypothetical protein	Chromosome	114	140	3.96E-06
yciR	RNase II stability modulator	Chromosome	18	2	4.23E-06
rbsK	Ribokinase	Chromosome	52	52	9.08E-04
yfiR	Hypothetical protein	Chromosome	32	23	9.08E-04
ydiV/nlpC	Hypothetical protein/	Chromosome	12	1	9.08E-04
fimD	Outer membrane usher protein FimD precursor	Chromosome	0	54	8.63E-03
		Total reads	41,375	107,967	

Table 5.10 25°C biofilm vs 25°C static control read number comparison

A proportion test was carried out in R in order to compare the number of reads for each gene and determine the probability that these proportion differ. All genes with significant difference between the number of reads present in the 25°C biofilm and the 25°C static control are displayed in this table. Where two genes are displayed separated by a forward slash (/) this represents an intergenic insertion between the two named genes. The total number of mapped reads for each library is displayed in the bottom row.

Gene	Function	Location	25°C Biofilm (reads per gene)	25°C static control (reads per gene)	P value
fimE	Tyrosine recombinase	Chromosome	147	198	3.46E-06
rbsR	Transcriptional repressor RbsR	Chromosome	80	107	4.85E-03
fimD	Outer membrane usher protein FimD precursor	Chromosome	0	58	4.85E-03
yfiR	Hypothetical protein	Chromosome	32	25	4.85E-03
yjcC	Hypothetical protein	Chromosome	114	171	4.85E-03
yciR	RNase II stability modulator	Chromosome	18	5	5.46E-04
		Total reads	41,375	108,554	

5.5 Discussion

Biofilm formation has been shown, both in previously published data and in this thesis, to be dependent on type 1 pili *in vitro* at 25°C. However, this thesis has also shown that at 37°C, biofilm formation in the same *in vitro* assay is type 1 pili independent. This type 1 pili independence in biofilm formation has also been shown to be relevant to CAUTI in a mouse model. In order to further understand this type 1 pili independence a TraDIS screen was carried out in UTI89 with the aim of elucidating the mechanism by which biofilms are able to form *in vitro at* 37°C.

Due to a number of unforeseen technical issues, there was a shortfall in the number of reads required to be able to make strong conclusions. Despite this, some useful information was gleaned from this TraDIS experiment making it a useful proof of concept experiment. Several other limitations in this study are also likely to have affected the data obtained and as a result caution was required when drawing conclusions from this TraDIS dataset.

The major issue encountered was a lack of diversity at the beginning of each read in TraDIS libraries. This resulted in poor cluster identification during the sequencing run and therefore a large reduction in reads compared to the expected Hiseq 4000 output. This could be overcome in future experiments by sequencing a mixture of TraDIS and whole genome libraries in each run, rather than whole runs of TraDIS libraries. This lack of diversity may have been exacerbated by the positioning of the transposon specific primer within the transposon cassette when amplifying transposon insertion junctions. In this case the primer position resulted in a longer transposon sequence being present in each read and reduced the length of genomic sequence generated. Moving this primer closer to the end of the transposon may help to reduce the lack of diversity in the sequencing library. Although, in this case however, the extra transposon sequence present in the reads was used to perform a second demultiplexing step. This was carried out due to high levels of nonspecific random priming (of the transposon specific library amplification primer) across the genome. In

order to overcome this mis-priming in future experiments it would be beneficial to carry out nested PCR in order to correctly amplify transposon specific sequencing library fragments.

Another major limitation of this TraDIS experiment is the low number of individual insertions present in the transposon mutant library. Despite the generation of a library of 320,000 successful transformants this corresponded to only 42,205 individual insertions (detected by TraDIS), resulting in an unsaturated mutant library. In order to improve this, more UTI89 Tn5 transformants should be generated, and could be achieved by increasing the competency of the cells used for mutagenesis and by pooling several mutant batches.

Despite normalising by gene length a heavy insertion bias amongst the data is evident. There are many more reads and individual insertions about the origin of replication compared to the terminus (Figure 5.3). A similar pattern of insertion bias has been reported for other TraDIS data sets and is due to ongoing chromosomal and plasmid replication. In bacteria, replication occurs about a replication fork and results in multiple copies of the DNA towards the origin in a single cell at any one time. This means that during transformation there are more copies toward the origin resulting in a higher number of insertions here compared to around the terminus (Chao et al., 2016). A computational tool for normalising this bias has been developed by Zomer et al. (2012).

Prior to TraDIS screening the mutant library was grown overnight (for 16 hours), meaning that the cells had undergone 50 generations before the biofilm screening assays and the mutant library was sequenced. This poses a further problem for the data produced in this TraDIS experiment as the growth kinetics of all mutants is unlikely to be even. This may result in certain mutants expanding more rapidly in the population and being over represented in the mutant library.

Validation of TraDIS libraries usually involves the assessment of genes essential for normal growth of the bacterial strain in question. However, in this case the number of individual insertion mutants was so low that 62.3% of genes in UTI89 did not contain a single insertion. If essential genes were to be determined in this dataset the number of essential genes would be greatly

overestimated and differentiation of true essential genes, that contained insertions but were lost from the population due to a fitness defect in the test growth conditions, from those that never contained an insertion, would not be possible. Instead, the number of reads obtained for a list of genes previously reported to be essential for *E. coli* strains BW25113 and EC958, were determined.

Of the orthologs found for BW25113 essential genes, 12.1 % had reads present in the mutant library. Whilst for the majority a low number of reads were present, *tnaB* contained 204 reads and *bscB* contained 100. However, the list of essential genes for BW25113 was determined by the targeted deletion of single genes, where a mutant was not obtained these genes were listed as essential candidates. Therefore, it is possible that several of the genes in this list are not truly essential, but that due to technical and experimental reasons a mutant was not able to be made. This is likely the case for *tnaB* (A typtophan transporter), as successful mutation and deletion of these gene in *E. coli* has been reported elsewhere (Li and Young, 2013, Yanofsky et al., 1991). Also, *tnaB* has been shown to be essential for growth with tryptophan as the only carbon source but not in LB (Yanofsky et al., 1991). However, despite *bscB* (involved in cellulose synthesis) having been shown not to be essential for *E. coli* strain MG1655 in enriched LB media (Gerdes et al., 2003), there are no reports of this being true for growth in un enriched LB as was used in this TraDIS experiment.

Of the orthologs found for EC958 essential genes a low percentage (4.2%) were found to have reads present the mutant library and only 2 of these genes contained more than one read, *psd* and UTI89_C3454. The *psd* gene, encoding a phosphatidylserine synthase, has previously been deleted in *E. coli* strain RA2000 with the mutant strain growing in LB but displaying reduced swarming and motility (Shi et al., 1993) indicating that it is not essential in this strain, and this may also be the case for UTI89. UTI89_C354 contained the most reads at 14, however this is less reads than were detected in this gene by (Phan et al., 2013), who reported it as essential with 42 reads as it fell below the insertion index cut off value applied in their analysis. However, this gene has been reported as nonessential in another *E.* coli strain. Whilst UTI89_C3454 is a hypothetical gene in UTI89 its ortholog in *E. coli* strain RS218 is *hcp1* which is involved in a type VI secretion system

and has been successfully deleted in this strain with the mutant strain showing no growth defect in LB media compared to WT (Zhou et al., 2012), indicating that it may not be essential for growth in UTI89. Overall, due to the low number of reads and individual insertions, it cannot be concluded that those genes containing no reads were truly essential for growth in UTI89. With a more complex mutant library and higher sequencing depth the number of reads may be detected in these genes. However this information gives some indication of the validity of the data collected.

Despite these limitations, several genes of potential interest were identified in the TraDIS analysis that had a significantly lower number of reads compared to the 25°C biofilm and the mutant input library, and looked to potentially be important in biofilm formation at 37°C. These include *rbsR, rbsK, yjcC, rfaJ, rfaQ, ybhJ, yhiX, waaL* and *waaV,* several of which encode putative or hypothetical proteins. Although their exact functions have not been previously elucidated some of these genes of interest are in operons with other genes that have previously been shown to play a role in biofilm formation, (gene locations within operons determined using EcoCyc (Keseler et al., 2013)). Of particular interest - two genes from a ribose metabolism operon, *rbsK* and *rbsR* (Hope et al., 1986), were found to have a low number of reads in biofilms at 37°C compared to those at 25°C and therefore may potentially be involved in type 1 pili independent biofilm formation at 37°C. These genes have not previously been implicated in biofilm formation. The genes, *rfaJ, rfaQ, waaL and waaV* are all members of the major core-oligosaccharide assembly operon in *E. coli.* Although these genes have not been directly linked to biofilm formation in UPEC, other genes in this region, and LPS in general, have previously been implicated in biofilm formation (Nakao et al., 2012, Niba et al., 2007, Hadjifrangiskou et al., 2012). The involvement of LPS in biofilm formation however, is extremely complex and the exact mechanism behind this is yet to be established.

The detection of *ybhJ* is interesting as although it encodes a hypothetical protein in UTI89, in K-12 it is predicted to be a hydratase and has been shown to be induced upon expression of *bola,* which has in turn been implicated in biofilm formation *in vitro* at 37°C (Dressaire et al., 2015). The transcriptional regulator GadX (encoded by *yhiX)*, is involved in acid sensitivity (Sayed et al., 2007)

and was detected in this screen as being potentially involved in 25°C biofilm formation. This gene has previously been shown to play a role in *E.coli* biofilm formation, with cells lacking this gene displaying a less robust biofilm structure. It has also been shown to be associated with biofilm related microarray data from several studies (Hodges et al., 2010). Again, although this gene has been implicated in biofilm formation, it is unclear exactly how it is involved in this process.

Several other genes relevant to biofilm formation at 25°C were also deemed to be significant in comparisons between TraDIS test conditions. Of particular note is the detection of a significantly lower number of reads (0 reads) in *fimD* in the 25°C biofilm TraDIS sample compared to both the mutant library input and the 37°C biofilm. Less reads were also detected for type 1 pili for the 37°C biofilm compared to the input library, however, this was likely due to the fact that there were more than double the number of reads in the mutant library than the 37°C biofilm sample in total. The two biofilm samples, 25°C and 37°C, have very similar numbers of reads and the difference in the number of reads from *fimD* for each was extremely significant ($p=7.52\times10^{-6}$). A significant difference was also detected in the number of reads for *fim E* in a comparison between the mutant library input and the 25°C biofilm, as well as between the 25°C and 37°C biofilm samples. In this case however there were significantly more reads in the 25°C biofilm suggesting that this gene is dispensable for biofilm formation at 25°C. Of the structural and assembly proteins constituting the type 1 pilus, *fimD* is the only one of significance detected as being required for biofilm formation at 25°C with zero insertions. There were more reads detected for *fimD* than the other components of the *fim* operon in all samples, aside from the 25°C biofilm. The fact that no *fimD* insertions were detected in the 25°C biofilm despite there being more insertions in *fim D* overall strengthens the interpretation that this gene is required for biofilm formation at 25°C and that this can be detected via TraDIS. There may be more reads/insertions for *fimD* in general because it is much longer than the rest of the type 1 pili components with a length of 878bp, whilst the next largest component is the *fimH* adhesin at 300bp. This may have been true for a transposon screen previously carried out by (Hadjifrangiskou et al., 2012), which also seemed to detect more

insertions in the *fimD* gene than other type 1 pili components, although this wasn't explicitly described or commented on by the authors.

Like *fimD, fimE* is part of the *fim* operon encoding type 1 pili, however *fimE* is a tyrosine recombinase responsible for regulation of type 1 pilus expression by phase variation. *fimE* is one of several recombinases that affect the phase variation of the *fimS* element, which contains the promoter for *fimA* – the major structural component of the pilus, however this particular one is responsible for switching cells from phase on to phase off (Gally et al., 1996). In an environment where type 1 pili are required, for example the 25°C biofilm, the *fimE* gene is therefore not required.

Taken together, these data for *fim D* and *fimE* support previously published data as well as data presented in this thesis that have shown biofilm formation to be dependent on type 1 pili at 25°C. It also shows that despite the low number of reads generated in this experiment, differences in relevant genes can be detected, validating this TraDIS dataset. Although caution must still be taken when making subsequent observations, this also allows more confidence in the interpretation of other genes that may be involved in biofilm formation and more specifically, type 1 pili independent biofilm formation at 37°C.

Several genes in the K1 capsule gene cluster were also identified as having a significantly higher number of reads in the 37°C biofilm compared to the input mutant library and the 25°C biofilm suggesting that they are not required for biofilm formation at 37°C. These genes were: *neuD, neuB, neu* and *kspM.* The expression of this gene cluster has previously been shown to be controlled by several global regulators that are temperature regulated and capsule genes are known to be preferentially expressed at 37°C (Orskov et al., 1984). However, despite being important for IBC maturation (Anderson et al., 2010, Goller and Seed, 2010), there is strong evidence showing that the expression of capsules attenuates biofilm formation at 37°C *in vitro.* This is thought to be via a shielding mechanism where the expressed capsule physically shields other surface adhesins (Hanna et al., 2003) and this has been shown to be true for Ag43 (Schembri et al., 2004).The fact that observations from this TraDIS experiment are consistent with previous research is another

indication of how useful this data may be if more reads were obtained, and again gives a good basis in order to interpret the role of other genes that may be important for biofilm formation at 37°C.

As previously mentioned, there is a shortfall in the number of reads required for a dataset of this kind. This may be the cause of the low number of significant differences detected in pairwise comparisons of read numbers. Previous studies have suggested that there may be hundreds of genes involved in biofilm formation and as such more would be expected from this kind of high-throughput screen. However, no nonspecific filtering was performed in order to remove reads that were present only once in the dataset. Had this been performed the corrected q- values (two tailed proportion test corrected for multiple comparisons using the Benjamin & Hochberg approach) were likely to be decreased as less comparisons would be corrected for potentially resulting in more significant differences being detected.

The nature of the biofilm assay used for this TraDIS experiment, the micro titre plate assay, is likely to limit this experiment to the study of factors involved in in the initiation of biofilm formation rather than maturation. That being said, biofilms in this assay have been shown to exhibit some features of mature biofilm (O'Toole, 2011). This TraDIS screen and phenotypic validation could subsequently be performed using more sophisticated biofilm assays, such as the flow cell model, in order to further understand the later stages of biofilm formation and maturation. Despite this potential limitation the study of biofilm initiation is still extremely important in order to further understand how and why biofilms form in the clinical setting and in order to prevent and treat biofilm mediated infections such as CAUTI.

5.6 Further work

As previously mentioned, due to several issues encountered in the TraDIS sequencing process the sequencing data generated was not sufficient to be able to draw strong conclusions regarding the mechanism behind type 1 pili independent biofilm formation at 37°C from the TraDIS data set presented in this thesis. From the data obtained however, some reasonable avenues for further

exploration are able to be drawn. In order to strengthen these conclusions, and gain a clearer insight into biofilm mechanisms, this TraDIS experiment should be repeated, taking the previously experienced issues into account in order to generate a much larger, more accurate, dataset and be able to perform a more comprehensive analysis.

In order to ascertain whether the gene targets identified by TraDIS analysis are responsible for biofilm formation at either temperature it is important that these are validated *in vitro* and possibly in *vivo.* The generation of clean knockout strains of genes of interest, such as *rbsR, rbsK, yjcC, rfaJ, rfaq, ybhJ, yhiX, waaL* and *waaV,* and their subsequent screening using the CV microtitre biofilm assay should be carried out in order to validate the involvement of these genes and their products in UPEC biofilm formations *in vivo* at both 25°C and 37°C.

Mutants of particular interest that display a reduced biofilm phenotype at 37°C, indicating that the deleted gene is required for biofilm formation at this temperature *in vitro* should then be screened *in vivo* in order to determine their relevance in biofilm formation and infection in a mouse model for both UTI and CAUTI.

Chapter 6: Final Discussion

6.1 Discussion

UTIs and CAUTIs are extremely common infections (Foxman, 2002) and can have potentially fatal consequences (Ministry of Health Singapore., 2014, Office for National Statistics., 2015). These infections are a major healthcare burden (Foxman et al., 2000a) and act as a significant reservoir for antibiotic resistance (Tambyah and Maki, 2000a). It is therefore of utmost importance that these infections are well understood and characterised in order to inform effective treatment and prevention strategies.

UPEC strains (the most common causative agents of both UTIs and CAUTIs (Flores-Mireles et al., 2015) have a number of virulence factors that aid their pathogenesis. One major group of these virulence factors are those that contribute to biofilm formation. Biofilm formation is known to be particularly important in UTI pathogenesis, which is mediated by intracellular biofilms known as IBCs (Anderson et al., 2003). Biofilms have also been shown to be present on the bladder surface (Mulvey et al., 1998) and they are known to be the major cause of CAUTIs by forming on the catheter surface. There is strong evidence to show that IBC formation is dependent on type 1 pili (Martinez et al., 2000, Wright et al., 2007) and as such they are also thought to be important in CAUTIs however this is comparatively under studied (Jacobsen et al., 2008).

In the laboratory there are several ways in which this type 1 pili mediated biofilm formation can be modelled, both *in vitro* and *in vivo.* A well characterised mouse model exists for UTIs caused by UPEC (Hung et al., 2009) and *in vitro, s*everal different biofilm models can be used to study type 1 pili mediated biofilm formation. The crystal violet microtitre assay is often used *in vitro* due to its simplicity and high throughput nature. This assay is commonly used at 25°C when studying type 1 pili however the incubation temperature used varies amongst publications (Hadjifrangiskou et al., 2012, Agarwal et al., 2013, Khanal et al., 2015). When used at 25°C biofilm formation is type 1 pili dependent (Pratt and Kolter, 1998) and corresponds directly to *in vivo* data showing infection and

IBC development to be dependent on type 1 pili (Wright et al., 2007). These IBC structures have also been identified in the urine of human UTI patients (Rosen et al., 2007) confirming their relevance to human infection. This availability of a set of corresponding *in vitro* and *in vivo* tools that accurately reflect the infection biology is invaluable for the study UTIs. A well-integrated set of models however, does not exist for the study of CAUTI. Published studies have shown that the presence of a catheter implant in a mouse model for UTI affects the pathogenicity of uropathogenic bacteria such as *E. faecali*s (Guiton et al., 2010) indicating that the pathogenesis of CAUTI and UTI may differ more than previously anticipated. Alongside this difference in pathogenesis of CAUTI and UTI in *in vivo* models, a previous observation in the Chen lab (Majid Eshaghi & Swaine Chen, personal communication, September 2013) suggested that the *in vitro* biofilm formation phenotype of UPEC differed with the incubation temperature.

In order to successfully study the difference in UTI and CAUTI pathogenesis and the involvement of biofilms in these infections it is important that the laboratory models used, and how they relate to infection, are fully understood. The initial aim of this thesis was therefore to investigate and characterise the potential difference in biofilm formation of type 1 pili deficient UTI89 at varying temperatures. This was in order to address the question of whether biofilm formation *in vitro* is type 1 pili dependent at 37°C (a more physiologically relevant temperature) and to assess the *in vivo* relevance of this biofilm phenotype to CAUTI. To achieve these aims various different approaches were utilised including, *in vitro* phenotypic characterisation, *in vivo* characterisation in a murine model of CAUTI, and a genetic approach to characterisation.

This thesis supports previously published data that type 1 pili are required for biofilm formation at 25°C in an *in vitro* biofilm assay (Pratt and Kolter, 1998, Hadjifrangiskou et al., 2012). However, a major finding of this thesis is that type 1 pili are not required for biofilm formation in the same *in vitro* biofilm assay at 37°C – a finding which has been confirmed using gene knockouts, manipulation and complementation as well as with chemical inhibition studies (Chapter 3). Type 1 pili independent biofilm formation was only observed at 37°C; this observation is particularly

interesting and relevant to CAUTI as when in clinical use sections of a urinary catheter are maintained at temperatures varying from the collection bag that may be at room temperature (approximately 25°C), or slightly warmer if strapped to the leg of a patient, up to 37°C where the catheter tip rests directly in urinary tract. The varying dependence of UPEC on type 1 pili for biofilm formation raises the possibility that UPEC may be able to take advantage of a particular niche along a catheter creating a foothold for infection where they may not have previously been able to. This hypothesis is supported by data from a mouse model of CAUTI caused by *Enterococcus faecalis* (Guiton et al., 2010). *E. faecalis* causes little to no infection in a mouse model for UTI however in the presence of a catheter implant in a CAUTI model it is able to colonise mouse bladders. As such, the relevance of the type 1 pili independent biofilm formation, at 37°C *in vitro,* to CAUTI was sought. In order to elucidate any correspondence between the type 1 pili independent 37°C microtitre biofilm model and CAUTI, the ability of UPEC strains to cause infection in a murine CAUTI model was tested with and without the presence of functional type 1 pili.

Like E. *faecalis*, type 1 pili deficient UPEC strains (both UTI89 and CFT073) exhibited an increase in their ability to infect mouse bladders in the CAUTI model compared to the standard UTI mouse model, and although this was a modest increase, it was statistically significant. The same type 1 pili deficient UPEC strains were also able to colonise silicone catheter implants. This ability of type 1 pili deficient UPEC strains to adhere to catheter implants and infect mouse bladders corresponds to the *in vitro* CV model at 37°C (whereby type 1 pili are not required for biofilm formation) and again suggests that the pathogenesis of CAUTI and UTI differ more than has been previously suggested.

Despite having defined an alternative, previously uncharacterised, mode of biofilm formation *in vitro*, and established correspondence of this phenotype to type 1 pili independent infection and colonisation in a mouse model of CAUTI (another previously uncharacterised phenotype), the exact mechanism of this biofilm formation was not yet established. In order to determine the mechanism behind this type 1 pili independent biofilm formation a genomics based approach was utilised.

Biofilm formation in UPEC has been shown to be an extremely complex process. Several major biofilm factors have been identified including type 1 pili, however multiple studies have shown that hundreds of different genes may be involved in *E. coli* and UPEC biofilms formation (Niba et al., 2007, Hadjifrangiskou et al., 2012). As such, a high-throughput, genome wide screening approach was required in order to identify potential biofilm factors involved in the type 1 pili independent biofilm formation identified in this thesis, to this end TraDIS (Langridge et al., 2009) was the technique of choice.

TraDIS screening was carried out in the context of the *in vitro* model rather than the *in vivo* CAUTI model because a previous study identified and characterised a substantial population bottleneck in the *in vivo* model of UTI (Schwartz et al., 2011). It was hypothesised that the bottleneck in biofilm formation *in vitro* would be less substantial and this was confirmed using a competitive biofilm formation experiment. Prior to carrying out this TraDIS screen it was important to characterise potential population bottlenecks in the microtitre plate assay to be used to screen transposon mutants. It was determined that biofilms in this particular assay were not subject to a major population bottleneck caused by the founder effect. Kanamycin resistant cells were recovered from mixed biofilm populations even when their starting concentration was as little as 0.0001% of the inoculum (2 CFU/200 µl). This result is important as it confirms there is unlikely to be any major loss of diversity as a result of the library screening conditions.

A saturated mutant library of 320,000 transposon mutants was generated for this thesis and was, at the time of writing, to the best of the authors knowledge, the most complex mutant library described in this particular UPEC strain, although not for *E. coli* in general (Phan et al., 2013, Subashchandrabose et al., 2013). This complex library of mutants will be very useful for the further study of UTI and CAUTI pathogenesis.

This library of transposon mutants was passaged in the *in vitro* biofilm assay before being subject to TraDIS. Using this technique, sequencing of only the transposon insertion junctions with genomic DNA allows the identification of essential genes for the condition in which the mutant

library was passaged. Unfortunately, due to several technical issues the TraDIS data presented in this thesis is not robust enough to be able to make conclusions regarding genes that may, or may not, be essential for biofilm formation at 37°C. However, analysis of the TraDIS data in this thesis has uncovered several factors that may potentially be the cause of the type 1 pili independent biofilm formation identified both *in vitro* and *in vivo* in this thesis. These genes include several involved in core-oligosaccharide assembly and outer membrane stability, such as *rfaQ*, *rfaJ*, *waaL* and *waaV*. LPS and other genes in this region have previously been implicated in biofilm formation (Nakao et al., 2012, Niba et al., 2007, Hadjifrangiskou et al., 2012) although the exact mechanisms for this association are complex and have not been clearly established.

Also implicated in this screen was the transcriptional regulator GadX, encoded by *yhiX,* that is involved in acid sensitivity (Sayed et al., 2007) This gene has previously been shown to play a role in *E.coli* biofilm formation, with cells lacking this gene displaying a less robust biofilm structure and has been shown to be associated with biofilm related microarray data from several studies (Hodges et al., 2010). Again, although this gene has been implicated in biofilm formation, it is unclear exactly how it is involved in this process. Another particularly interesting finding from the TraDIS is the implication that several genes involved in ribose metabolism, *rbsK* and *rbsR* (Hope et al., 1986) may potentially be involved in type 1 pili independent biofilm formation at 37°C. These genes have not previously been implicated in biofilm formation and provide a further avenue for future exploration.

As mentioned previously, the TraDIS data presented in this thesis has several notable draw backs and so caution should be taken when interpreting these results. One such drawback was caused by low nucleotide diversity in the first 35 cycles of the sequencing run. This was due to the presence of the same transposons sequence in each read and lead to poor cluster calling and subsequently a reduced number of reads. Further to this, due to high levels of random priming of the transposon specific primer used to selectively amplify across the genome, the reads that were obtained from the sequencing run were subject to a second round of demultiplexing in order to

obtain true insertion junctions, however this resulted in a loss of around 95% of the total sequence reads. This issue of non-specific priming is important to address in future TraDIS experiments.

In order to be certain that genes identified in this study are in fact responsible for the type 1 pili independent biofilm formation at 37°C (as previously observed), this study would benefit from repeated sequencing of the TraDIS sequencing libraries addressing the issues previously described (as discussed in section 5.5) as well as the full validation of potential hits. In order to confirm any role in this biofilm formation clean knockout strains need to be generated and screened in the CV micro titre biofilm assay. Those genes of interest that are found to be required for *in vitro* biofilm formation at 37°C can then be studied in the context of the CAUTI mouse model.

6.2 Conclusions

The major aim of this project was to investigate and characterise the dependence of UPEC strains on type 1 pili for biofilm formation *in vitro* at varying temperatures, and to assess the relevance of type 1 pili to CAUTI *in vivo*. The specific aims for each chapter as previously described are listed again below alongside the major conclusions that were made in each section:

Chapter 3: Genetic Characterisation of a Temperature Dependent Biofilm

Aims: To validate and characterise the initial observation that biofilm formation by UTI89 varies with temperature (Majid Eshaghi & Swaine Chen, personal communication, September 2013) and to verify this phenotype using different attachment surfaces, genetic mutants and chemical inhibition studies.

Conclusions:

- UTI89 biofilm formation was type 1 pili independent at 37°C (confirmed genetically and with chemical inhibition studies).
- UTI89 was dependent on type 1 pili for biofilm formation at other temperatures (20°C, 25°C, 30°C and 42°C).
- Type 1 pili independent biofilm formation was observed at 37°C on both PVC and polystyrene adhesion surfaces.
- CFT073 also exhibited type 1 pili independent biofilm formation at 37°C but not at 25°C.
- MG1655 is dependent on type 1 pili for biofilm formation at both 25°C and 37°C.

Chapter 4: *In Vivo* Characterisation of Type 1 Pili Independent Biofilm Formation

Aims: To assess the *in vivo* relevance of varying biofilm formation mechanisms by testing the ability of type 1 pili deficient UTI89 to cause infection *in vivo,* in a murine model for CAUTI, and to elucidate any correspondence between biofilm formation *in vitro* and the *in vivo* CAUTI model.

Conclusions:

- Type 1 pili are required for UTI89 and CFT073 bladder infection in a mouse model for standard UTI.
- Type 1 pili did play a role in a mouse model for CAUTI but were not essential for bladder and catheter colonisation in this model.
- UTI89Δ*fim* was able to colonise catheter implants and mouse bladders in the presence of a catheter.
- UTI89 did not form IBCs in the absence of type 1 pili, even in catheterised mice.
- The ability of UPEC to infect mouse kidneys was not affected by the presence of a catheter implant.

Chapter 1:

To genetically characterise the variation in biofilm formation mechanism at different temperatures. To utilise transposon directed insertion site sequencing (TraDIS) with a view to identifying causative genes responsible for biofilm formation *in vitro* at both 25°C and 37°C.

Conclusions:

- In order to make valid conclusions with regards to factors required for biofilm formation at various temperature several limitations and issues need to be addressed and the TraDIS screen repeated. Including, but not limited to, the complexity of the mutant library, sequencing library diversity, non-specific primer binding, insertional bias.

6.3 Future directions

To further understand the role of type 1 pili independent biofilm formation in CAUTI there are several avenues that could be further explored. One such area would be the characterisation of biofilm formation of clinical isolates from CAUTI patients. The strains used in this thesis, although well studied and prototypic, are isolates from uncomplicated UTIs and therefore do not necessarily best represent CAUTI. As part of this PhD a clinical sample collection was planned in the form of a broader CAUTI microbiome study. The aim of this study was to collect matched catheter, urine and faecal samples from hospitalised patients presenting with CAUTI in order to study the host source/sink dynamics with a view to better understanding CAUTI pathogenesis. The clinical study was planned and the ethical approval sought, although due to time constraints this project was not followed in detail as part of this thesis. Sample collection is, however, now underway and CAUTI isolates collected as a part of this clinical study may be used in order to study the type 1 pili independent biofilm formation at 37°C phenotype previously described in a more relevant CAUTI setting.

Another way in which this type 1 pili independent biofilm formation could be characterised is using RNAseq. Alongside the genetic TraDIS screen presented in this thesis, RNAseq would provide further insight into the genetic regulation of this phenotype. High throughput RNAseq would allow the understanding of this biofilm phenotype, and possibly CAUTI pathogenesis, at a transcriptome level. This would allow the identification of any expression differences that may be responsible for the type 1 pili independent biofilm formation phenotype that may not be identifiable from a high throughput screen at the gene level, such as TraDIS.

The current phenotypic analysis of the *in vitro* type 1 pili independent biofilm formation presented in this thesis is limited to the assessment of biofilm formation in a CV assay. Microscopic investigation of biofilms would allow the analysis of any structural differences between biofilms formed at 25°C and 37°C. Confocal imaging in particular would be particularly useful to study the

3D structure of biofilms formed at various temperatures. This *in vitro* characterisation could also be followed up by the assessment of biofilms formed at each temperature, 25°C and 37°C., for a secondary phenotype, for example resistance or sensitivity to either antibiotic or DNase treatment. The susceptibility of the biofilm to DNase may be altered if a change in the biofilm formation mechanism causes a change in the levels of extracellular DNA, a component of the ECM. Similarly, biofilms are known to confer resistance to antibiotics (see Introduction section 1.4.2.4) and so a change in the mechanism of formation or structure of the biofilm may lead to a change in antibiotic susceptibility. A secondary antibiotic resistance phenotype such as this could have clinical implications and inform the treatment/management of such infections. This is a particularly important area of research due the emergence of antibiotic resistant, a significant issue when treating biofilm mediated infections such as UTI and CAUTI. Further elucidation of the type 1 pili independent biofilm formation mechanism of UPEC will aid in the understanding of CAUTI pathogenesis, potentially informing research into the prevention, management and cure of CAUTI.

6.4 Concluding remarks

This thesis presents and characterises a previously unreported biofilm formation phenotype that does not require type 1 pili both *in vitro* at 37°C and *in vivo* in a mouse model for CAUTI. High throughput genomic screening has also provided insight into the potential mechanisms behind this alternative mode of biofilm formation. This correspondence between *in vitro* and *in* vivo CAUTI is extremely important for the study of CAUTI pathogenesis. This information combined, furthers current research into CAUTI providing a potential, novel mechanism by which these infections occur. CAUTI research has previously overlapped with that of UTIs which is historically very well characterised although the exact overlap in mechanisms has not previously been established (Mobley et al., 1987, Jacobsen et al., 2008).

CAUTIs are common infections and are a major source of antibiotic resistant pathogens in hospitals (Tambyah and Maki, 2000a) and as such it is important that these infections are managed effectively. At present the best treatment strategies are crude and generally involve the removal of

infected catheters followed by treatment with antibiotics when necessary. Whilst this is generally effective, it can be a major source of discomfort to patients and recurrent infection may still occur (Tenney and Warren, 1988). Additionally, antibiotic treatment in catheterised patients increases the risk of development of antibiotic resistance. Further study of this alternative biofilm formation mechanism will benefit future CAUTI research. A clear understanding of CAUTI pathogenesis and how it differs to that of uncomplicated UTI is extremely important.

Overall this thesis contributes to the field of CAUTI pathogenesis – an area which has in the past been overlooked compared to standard UTIs (Jacobsen et al., 2008).The data presented in this thesis contributes to the understanding of how the pathogenesis of UTI and CAUTI differ, presents a set of models that can be used for this purpose (Figure 4.1). This information, along with further characterisation, will help to inform further research into UTI and CAUTI, vaccine targets for the prevention of these infections, as well as into other, more effective, prevention and treatment strategies in the long run.

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Appendix A ***P* values for all animal experiments**

A.1 ***P* values for bladder titres (Mann Whitney U test)**

	E. faecalis -	*E. faecalis* +	WT UTI89 -	WT UTI89 +	UTI89Δ*fim* -	UTI89Δ*fim* +	WT CTF073 -	WT CTF073 +	CFT073Δ*fim* -	CFT073Δ*fim* +
E. faecalis -		<0.0001	<0.0001	<0.0001	0.7413	0.0038	<0.0001	<0.0001	0.0086	0.7860
E. faecalis +			<0.0001	0.857	<0.0001	<0.0001	0.6077	0.9025	<0.0001	<0.0001
WT UTI89 -				<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
WT UTI89 +					<0.0001	<0.0001	0.3881	0.7078	<0.0001	<0.0001
UTI89Δ*fim* -						0.0010	<0.0001	<0.0001	0.0014	0.9084
UTI89Δ*fim* +							<0.0001	<0.0001	<0.0001	0.0213
WT CTF073 -								0.7691	<0.0001	<0.0001
WT CTF073 +									<0.0001	<0.0001
CFT073Δ*fim* -										0.0064
CFT073Δ*fim* +										

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ns

A.2 *P* values for Kidney titres (Mann Whitney U test)

	E. faecalis -	*E. faecalis* +	WT UTI89 -	WT UTI89 +	UTI89Δ*fim* -	UTI89Δ*fim* +	WT CTF073 -	WT CTF073 +	CFT073Δ*fim* -	CFT073Δ*fim* +
E. faecalis -		0.0008	0.3271	0.3003	0.0824	0.1329	0.0011	<0.0001	0.8613	0.3422
E. faecalis +			0.0006	0.0007	0.0125	0.0066	0.3280	0.0889	0.0001	0.0016
WT UTI89 -				0.9902	0.3538	0.6215	0.0008	<0.0001	0.2296	0.8357
WT UTI89 +					0.4854	0.6120	0.0031	<0.0001	0.2725	0.7101
UTI89Δ*fim* -						0.8233	0.0066	<0.0001	0.0450	0.3511
UTI89Δ*fim* +							0.0140	0.0004	0.1410	0.4517
WT CTF073 -								0.6551	0.0007	0.0079
WT CTF073 +									<0.0001	0.0002
CFT073Δ*fim* -										0.2700
CFT073Δ*fim* +										

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ns

A.3 *P* values for Catheter titres (Mann Whitney U test)

	E. faecalis	WT UTI89	UTI89Δ*fim*	WT CTF073	CFT073Δ*fim*
E. faecalis		0.2367	0.0081	0.4351	0.0026
WT UTI89			< 0.0001	0.7534	< 0.0001
UTI89D*fim*				0.0052	0.2177
WT CTF073					0.0018
CFT073D*fim*					

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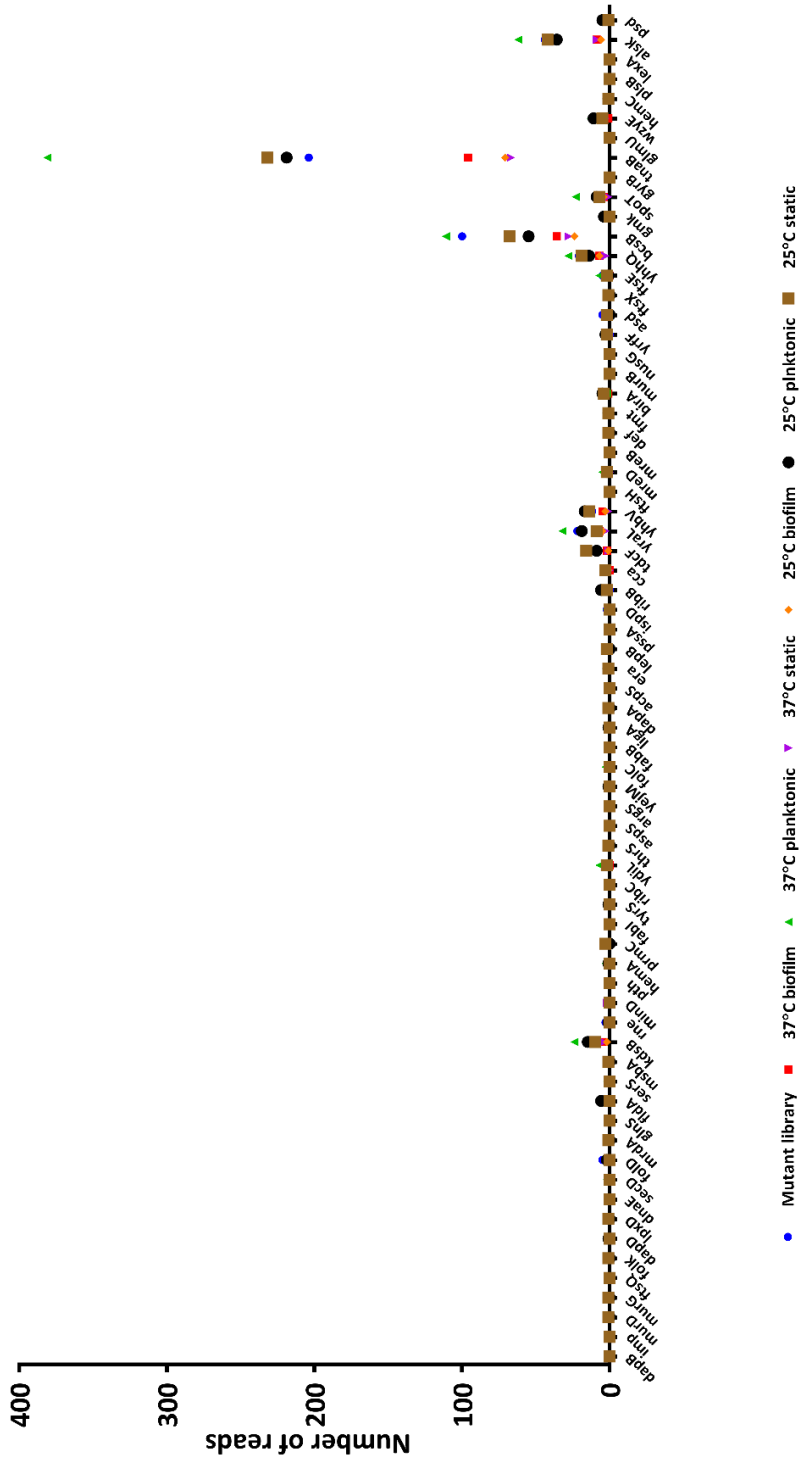
ns

Appendix B **Comparison of TraDIS data to Keio collection (BW25113) and EC958 essential genes.**

B.1 **Keio collection (*E. coli* K-12 strain BW25113)**



Number of reads obtained for essential genes in K-12 BW25113. Essential genes plotted if they contained at least one read in any of the 7 TraDIS libraries sequenced. See table overleaf for all orthologs including those with 0 reads in all libraries.

UTI89 Gene	MG1655 Gene	Gene name (Keio)	W3110 Gene	Mutant Library	37°C Biofilm	37°C Planktonic	37°C Static	25°C Biofilm	25°C Planktonic	25°C Static
UTI89_C0027	b0025	ribF	JW0023	0	0	0	0	0	0	0
UTI89_C0029	b0027	lspA	JW0025	0	0	0	0	0	0	0
UTI89_C0031	b0029	ispH	JW0027	0	0	0	0	0	0	0
UTI89_C0034	b0031	dapB	JW0029	0	0	0	1	0	0	0
UTI89_C0061	b0054	imp	JW0053	0	0	0	0	1	0	0
UTI89_C0092	b0083	ftsL	JW0081	0	0	0	0	0	0	0
UTI89_C0093	b0084	ftsI	JW0082	0	0	0	0	0	0	0
UTI89_C0094	b0085	murE	JW0083	0	0	0	0	0	0	0
UTI89_C0095	b0086	murF	JW0084	0	0	0	0	0	0	0
UTI89_C0096	b0087	mraY	JW0085	0	0	0	0	0	0	0
UTI89_C0097	b0088	murD	JW0086	0	0	0	0	0	0	1
UTI89_C0098	b0089	ftsW	JW0087	0	0	0	0	0	0	0
UTI89_C0099	b0090	murG	JW0088	0	0	0	0	0	0	1
UTI89_C0100	b0091	murC	JW0089	0	0	0	0	0	0	0
UTI89_C0102	b0093	ftsQ	JW0091	0	1	0	0	0	0	0
UTI89_C0103	b0094	ftsA	JW0092	0	0	0	0	0	0	0
UTI89_C0104	b0095	ftsZ	JW0093	0	0	0	0	0	0	0
UTI89_C0105	b0096	lpxC	JW0094	0	0	0	0	0	0	0
UTI89_C0106	b0097	secM	JW5007	0	0	0	0	0	0	0
UTI89_C0107	b0098	secA	JW0096	0	0	0	0	0	0	0
UTI89_C0139	b0126	can	JW0122	0	0	0	0	0	0	0
UTI89_C0156	b0142	folK	JW0138	2	0	0	0	0	0	1
UTI89_C0170	b0154	hemL	JW0150	0	0	0	0	0	0	0
UTI89_C0172	b0156	yadR	JW0152	0	0	0	0	0	0	0
UTI89_C0180	b0166	dapD	JW0161	0	0	0	0	0	1	0
UTI89_C0182	b0168	map	JW0163	0	0	0	0	0	0	0
UTI89_C0183	b0169	rpsB	JW0164	0	0	0	0	0	0	0
UTI89_C0185	b0170	tsf	JW0165	0	0	0	0	0	0	0
UTI89_C0186	b0171	pyrH	JW0166	0	0	0	0	0	0	0
UTI89_C0187	b0172	frr	JW0167	0	0	0	0	0	0	0
UTI89_C0188	b0173	dxr	JW0168	0	0	0	0	0	0	0
UTI89_C0189	b0174	ispU	JW0169	0	0	0	0	0	0	0
UTI89_C0190	b0175	cdsA	JW5810	0	0	0	0	0	0	0
UTI89_C0191	b0176	yaeL	JW0171	0	0	0	0	0	0	0
UTI89_C0192	b0177	yaeT	JW0172	0	0	0	0	0	0	0
UTI89_C0194	b0179	lpxD	JW0174	0	0	0	0	0	0	1
UTI89_C0195	b0180	fabZ	JW0175	0	0	0	0	0	0	0
UTI89_C0196	b0181	lpxA	JW0176	0	0	0	0	0	0	0
UTI89_C0197	b0182	lpxB	JW0177	0	0	0	0	0	0	0
UTI89_C0199	b0184	dnaE	JW0179	1	0	0	0	0	0	0
UTI89_C0200	b0185	accA	JW0180	0	0	0	0	0	0	0
UTI89_C0203	b0188	tilS	JW0183	0	0	0	0	0	0	0
UTI89_C0210	b0194	proS	JW0190	0	0	0	0	0	0	0
UTI89_C0388	b0369	hemB	JW0361	0	0	0	0	0	0	0

UTI89_C0430	b0408	secD	JW0398	0	0	2	0	0	0	0
UTI89_C0431	b0409	secF	JW0399	0	0	0	0	0	0	0
UTI89_C0436	b0414	ribD	JW0404	0	0	0	0	0	0	0
UTI89_C0437	b0415	ribE	JW0405	0	0	0	0	0	0	0
UTI89_C0440	b0417	thiL	JW0407	0	0	0	0	0	0	0
UTI89_C0443	b0420	dxs	JW0410	0	0	0	0	0	0	0
UTI89_C0444	b0421	ispA	JW0411	0	0	0	0	0	0	0
UTI89_C0497	b0470	dnaX	JW0459	0	0	0	0	0	0	0
UTI89_C0502	b0474	adk	JW0463	0	0	0	0	0	0	0
UTI89_C0503	b0475	hemH	JW0464	0	0	0	0	0	0	0
UTI89_C0553	b0524	lpxH	JW0513	0	0	0	0	0	0	0
UTI89_C0555	b0526	cysS	JW0515	0	0	0	0	0	0	0
UTI89_C0559	b0529	folD	JW0518	5	0	3	1	0	2	0
UTI89_C0583	b0583	entD	JW5085	1	1	2	2	0	1	1
UTI89_C0636	b0634	mrdB	JW0629	0	0	0	0	0	0	0
UTI89_C0637	b0635	mrdA	JW0630	0	0	0	0	0	0	1
UTI89_C0642	b0639	nadD	JW0634	0	0	0	0	0	0	0
UTI89_C0643	b0640	holA	JW0635	0	0	0	0	0	0	0
UTI89_C0644	b0641	rlpB	JW0636	0	0	0	0	0	0	0
UTI89_C0645	b0642	leuS	JW0637	0	0	0	0	0	0	0
UTI89_C0655	b0657	lnt	JW0654	0	0	0	0	0	0	0
UTI89_C0684	b0680	glnS	JW0666	0	0	1	0	0	0	0
UTI89_C0689	b0684	fldA	JW0671	7	1	4	0	0	6	0
UTI89_C0727	b0733	cydA	JW0722	0	0	0	0	0	0	0
UTI89_C0899	b0884	infA	JW0867	0	0	0	0	0	0	0
UTI89_C0901	b0886	cydC	JW0869	0	0	0	0	0	0	0
UTI89_C0905	b0890	ftsK	JW0873	0	0	0	0	0	0	0
UTI89_C0906	b0891	lolA	JW0874	0	0	0	0	0	0	0
UTI89_C0908	b0893	serS	JW0876	0	0	2	0	0	0	0
UTI89_C0982	b0911	rpsA	JW0894	0	0	0	0	0	0	0
UTI89_C0985	b0914	msbA	JW0897	0	0	0	0	0	0	1
UTI89_C0986	b0915	lpxK	JW0898	0	0	0	0	0	0	0
UTI89_C0989	b0918	kdsB	JW0901	10	4	24	4	2	15	10
UTI89_C0994	b0922	mukF	JW0905	0	0	0	0	0	0	0
UTI89_C0995	b0923	mukE	JW0906	0	0	0	0	0	0	0
UTI89_C0996	b0924	mukB	JW0907	0	0	0	0	0	0	0
UTI89_C1002	b0930	asnS	JW0913	0	0	0	0	0	0	0
UTI89_C1019	b0954	fabA	JW0937	0	0	0	0	0	0	0
UTI89_C1194	b1069	mviN	JW1056	0	0	0	0	0	0	0
UTI89_C1209	b1084	rne	JW1071	3	0	1	0	0	1	0
UTI89_C1210	b1085	yceQ	JW5154	0	0	0	0	0	0	0
UTI89_C1217	b1092	fabD	JW1078	0	0	0	0	0	0	0
UTI89_C1218	b1093	fabG	JW1079	0	0	0	0	0	0	0
UTI89_C1220	b1094	acpP	JW1080	0	0	0	0	0	0	0
UTI89_C1225	b1098	tmk	JW1084	0	0	0	0	0	0	0
UTI89_C1226	b1099	holB	JW1085	0	0	0	0	0	0	0
UTI89_C1244	b1116	lolC	JW5161	0	0	0	0	0	0	0

UTI89_C1245	b1117	lolD	JW5162	0	0	0	0	0	0	0
UTI89_C1246	b1118	lolE	JW1104	0	0	0	0	0	0	0
UTI89_C1260	b1131	purB	JW1117	0	0	0	0	0	0	0
UTI89_C1359	b1174	minE	JW1163	0	0	0	0	0	0	0
UTI89_C1360	b1175	minD	JW1164	0	0	1	2	1	0	0
UTI89_C1398	b1204	pth	JW1195	1	0	0	0	0	0	0
UTI89_C1401	b1207	prsA	JW1198	0	0	0	0	0	0	0
UTI89_C1402	b1208	ispE	JW1199	0	0	0	0	0	0	0
UTI89_C1403	b1209	lolB	JW1200	0	0	0	0	0	0	0
UTI89_C1404	b1210	hemA	JW1201	2	0	3	1	1	1	0
UTI89_C1405	b1211	prfA	JW1202	0	0	0	0	0	0	0
UTI89_C1406	b1212	prmC	JW1203	0	0	1	1	0	0	3
UTI89_C1409	b1215	kdsA	JW1206	0	0	0	0	0	0	0
UTI89_C1419	none	none	JW5190	0	0	0	0	0	0	0
UTI89_C1464	b1572	ydfB	JW1564	0	0	0	0	0	0	0
UTI89_C1543	b1274	topA	JW1266	0	0	0	0	0	0	0
UTI89_C1548	b1277	ribA	JW1269	0	0	0	0	0	0	0
UTI89_C1561	b1288	fabI	JW1281	1	0	0	0	0	0	0
UTI89_C1828	b1637	tyrS	JW1629	1	0	0	0	0	1	0
UTI89_C1853	b1662	ribC	JW1654	0	0	0	0	0	0	0
UTI89_C1881	b1689	ydiL	JW1679	3	0	7	0	1	2	2
UTI89_C1906	b1713	pheT	JW1703	0	0	0	0	0	0	0
UTI89_C1907	b1714	pheS	JW5277	0	0	0	0	0	0	0
UTI89_C1909	b1716	rplT	JW1706	0	0	0	0	0	0	0
UTI89_C1911	b1718	infC	JW5829	0	0	0	0	0	0	0
UTI89_C1912	b1719	thrS	JW1709	0	0	0	0	0	0	1
UTI89_C1934	b1740	nadE	JW1729	0	0	0	0	0	0	0
UTI89_C1975	b1779	gapA	JW1768	0	0	0	0	0	0	0
UTI89_C2001	b1807	yeaZ	JW1796	0	0	0	0	0	0	0
UTI89_C2070	b1866	aspS	JW1855	0	0	1	0	0	0	0
UTI89_C2080	b1876	argS	JW1865	1	0	0	0	0	0	0
UTI89_C2113	b1912	pgsA	JW1897	0	0	0	0	0	0	0
UTI89_C2291	b2017	yefM	JW5835	0	0	0	0	0	0	0
UTI89_C2388	b2114	metG	JW2101	0	0	0	0	0	0	0
UTI89_C2427	b2153	folE	JW2140	0	0	0	0	0	0	0
UTI89_C2466	b2188	yejM	JW2176	2	0	0	0	0	1	0
UTI89_C2512	b2231	gyrA	JW2225	0	0	0	0	0	0	0
UTI89_C2515	b2234	nrdA	JW2228	0	0	0	0	0	0	0
UTI89_C2516	b2235	nrdB	JW2229	0	0	0	0	0	0	0
UTI89_C2600	b2315	folC	JW2312	1	0	3	0	0	0	0
UTI89_C2601	b2316	accD	JW2313	0	0	0	0	0	0	0
UTI89_C2601	none	none	JW5379	0	0	0	0	0	0	0
UTI89_C2608	b2323	fabB	JW2320	0	0	1	0	0	0	0
UTI89_C2731	b2400	gltX	JW2395	0	0	0	0	0	0	0
UTI89_C2743	b2411	ligA	JW2403	0	0	0	0	0	1	0
UTI89_C2744	b2412	zipA	JW2404	0	0	0	0	0	0	0
UTI89_C2798	b2472	dapE	JW2456	0	0	0	0	0	0	0

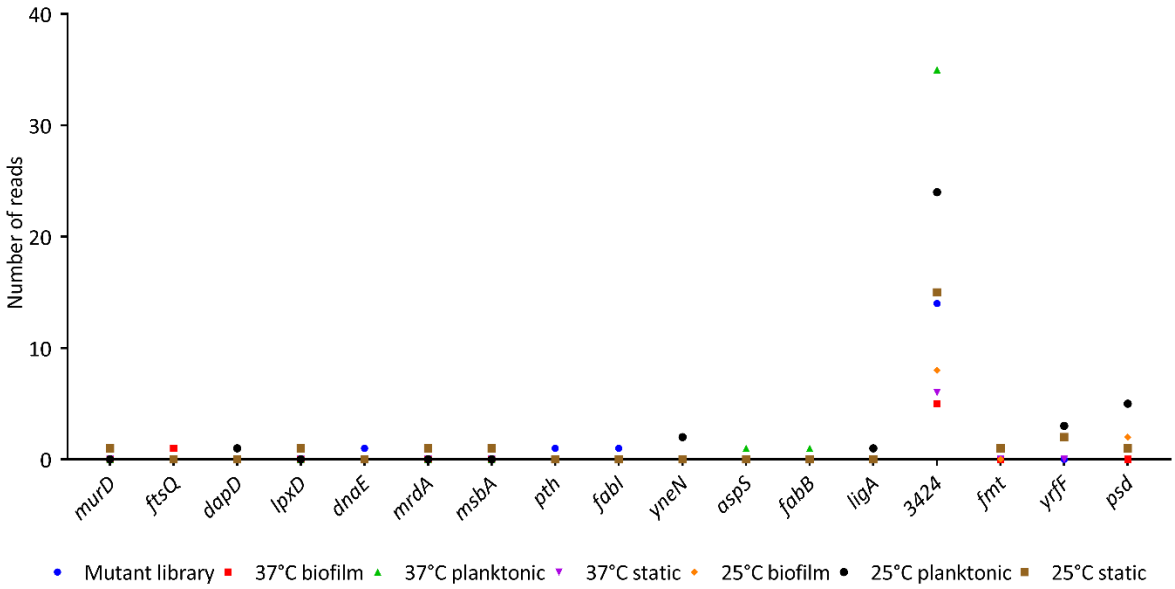
UTI89_C2805	b2478	dapA	JW2463	1	0	1	0	0	0	1
UTI89_C2832	b2511	der	JW5403	0	0	0	0	0	0	0
UTI89_C2835	b2514	hisS	JW2498	0	0	0	0	0	0	0
UTI89_C2836	b2515	ispG	JW2499	0	0	0	0	0	0	0
UTI89_C2855	b2533	suhB	JW2517	0	0	0	0	0	0	0
UTI89_C2879	b2559	tadA	JW2543	0	0	0	0	0	0	0
UTI89_C2884	b2563	acpS	JW2547	0	0	0	0	0	0	0
UTI89_C2887	b2566	era	JW2550	1	1	2	0	0	1	1
UTI89_C2888	b2567	rnc	JW2551	0	0	0	0	0	0	0
UTI89_C2890	b2568	lepB	JW2552	1	0	0	0	0	0	2
UTI89_C2895	b2573	rpoE	JW2557	0	0	0	0	0	0	0
UTI89_C2908	b2585	pssA	JW2569	0	0	0	0	0	0	0
UTI89_C2928	b2595	yfiO	JW2577	0	0	0	0	0	0	0
UTI89_C2939	b2606	rplS	JW2587	0	0	0	0	0	0	0
UTI89_C2940	b2607	trmD	JW2588	0	0	0	0	0	0	0
UTI89_C2942	b2609	rpsP	JW2590	0	0	0	0	0	0	0
UTI89_C2943	b2610	ffh	JW5414	0	0	0	0	0	0	0
UTI89_C2947	b2614	grpE	JW2594	0	0	0	0	0	0	0
UTI89_C2949	b2615	yfjB	JW2596	0	0	0	0	0	0	0
UTI89_C3004	b1145	ymfK	JW1131	0	0	0	0	0	0	0
UTI89_C3057	b2696	csrA	JW2666	0	0	0	0	0	0	0
UTI89_C3117	b2746	ispF	JW2716	0	0	0	0	0	0	0
UTI89_C3118	b2747	ispD	JW2717	2	0	2	0	0	0	0
UTI89_C3119	b2748	ftsB	JW2718	0	0	0	0	0	0	0
UTI89_C3148	b2779	eno	JW2750	0	0	0	0	0	0	0
UTI89_C3149	b2780	pyrG	JW2751	0	0	0	0	0	0	0
UTI89_C3153	b2783	chpR	JW2754	0	0	0	0	0	0	0
UTI89_C3230	b2828	lgt	JW2796	0	0	0	0	0	0	0
UTI89_C3308	b2925	fbaA	JW2892	0	0	0	0	0	0	0
UTI89_C3309	b2926	pgk	JW2893	0	0	0	0	0	0	0
UTI89_C3329	b2940	yqgD	JW2908	1	5	9	3	5	2	10
UTI89_C3331	b2942	metK	JW2909	0	0	0	0	0	0	0
UTI89_C3338	b2949	yqgF	JW2916	0	0	0	0	0	0	0
UTI89_C3444	b3018	plsC	JW2986	0	0	0	0	0	0	0
UTI89_C3464	b3030	parE	JW2998	0	0	0	0	0	0	0
UTI89_C3483	b3041	ribB	JW3009	0	1	2	1	1	6	2
UTI89_C3492	b3056	cca	JW3028	0	0	2	0	1	3	3
UTI89_C3500	b3064	ygjD	JW3036	0	0	0	0	0	0	0
UTI89_C3548	b3113	tdcF	JW5521	10	2	10	1	1	9	16
UTI89_C3573	b3146	yraL	JW3115	22	7	32	3	5	19	9
UTI89_C3586	b3159	yhbV	JW5530	12	5	15	1	3	17	14
UTI89_C3598	b3168	infB	JW3137	0	0	0	0	0	0	0
UTI89_C3600	b3169	nusA	JW3138	0	0	0	0	0	0	0
UTI89_C3610	b3178	ftsH	JW3145	0	0	0	0	0	0	0
UTI89_C3616	b3183	obgE	JW3150	0	0	0	0	0	0	0
UTI89_C3618	b3185	rpmA	JW3152	0	0	0	0	0	0	0
UTI89_C3620	b3186	rplU	JW3153	0	0	0	0	0	0	0

UTI89_C3621	b3187	ispB	JW3154	0	0	0	0	0	0	0
UTI89_C3623	b3189	murA	JW3156	0	0	0	0	0	0	0
UTI89_C3627	b3191	yrbB	JW5535	0	0	0	0	0	0	0
UTI89_C3635	b3199	yrbK	JW3166	0	0	0	0	0	0	0
UTI89_C3636	b3200	yhbN	JW3167	0	0	0	0	0	0	0
UTI89_C3660	b3230	rpsI	JW3199	0	0	0	0	0	0	0
UTI89_C3661	b3231	rplM	JW3200	0	0	0	0	0	0	0
UTI89_C3666	b3235	degS	JW3204	0	0	0	0	0	0	0
UTI89_C3679	b3249	mreD	JW3218	1	3	5	0	1	2	2
UTI89_C3680	b3250	mreC	JW3219	0	0	0	0	0	0	0
UTI89_C3681	b3251	mreB	JW3220	0	0	0	0	0	0	0
UTI89_C3688	b3255	accB	JW3223	0	0	0	0	0	0	0
UTI89_C3689	b3256	accC	JW3224	0	0	0	0	0	0	0
UTI89_C3727	b3282	yrdC	JW3243	0	0	0	0	0	0	0
UTI89_C3731	b3287	def	JW3248	1	0	1	0	0	0	1
UTI89_C3732	b3288	fmt	JW3249	1	0	1	0	0	1	1
UTI89_C3739	b3294	rplQ	JW3256	0	0	0	0	0	0	0
UTI89_C3740	b3295	rpoA	JW3257	0	0	0	0	0	0	0
UTI89_C3741	b3296	rpsD	JW3258	0	0	0	0	0	0	0
UTI89_C3742	b3297	rpsK	JW3259	0	0	0	0	0	0	0
UTI89_C3744	b3298	rpsM	JW3260	0	0	0	0	0	0	0
UTI89_C3746	b3300	secY	JW3262	0	0	0	0	0	0	0
UTI89_C3747	b3301	rplO	JW3263	0	0	0	0	0	0	0
UTI89_C3748	b3302	rpmD	JW3264	0	0	0	0	0	0	0
UTI89_C3750	b3303	rpsE	JW3265	0	0	0	0	0	0	0
UTI89_C3751	b3304	rplR	JW3266	0	0	0	0	0	0	0
UTI89_C3753	b3305	rplF	JW3267	0	0	0	0	0	0	0
UTI89_C3755	b3306	rpsH	JW3268	0	0	0	0	0	0	0
UTI89_C3756	b3307	rpsN	JW3269	0	0	0	0	0	0	0
UTI89_C3757	b3308	rplE	JW3270	0	0	0	0	0	0	0
UTI89_C3758	b3309	rplX	JW3271	0	0	0	0	0	0	0
UTI89_C3760	b3310	rplN	JW3272	0	0	0	0	0	0	0
UTI89_C3761	b3311	rpsQ	JW3273	0	0	0	0	0	0	0
UTI89_C3762	b3312	rpmC	JW3274	0	0	0	0	0	0	0
UTI89_C3764	b3313	rplP	JW3275	0	0	0	0	0	0	0
UTI89_C3765	b3314	rpsC	JW3276	0	0	0	0	0	0	0
UTI89_C3767	b3315	rplV	JW3277	0	0	0	0	0	0	0
UTI89_C3768	b3316	rpsS	JW3278	0	0	0	0	0	0	0
UTI89_C3770	b3317	rplB	JW3279	0	0	0	0	0	0	0
UTI89_C3771	b3318	rplW	JW3280	0	0	0	0	0	0	0
UTI89_C3773	b3319	rplD	JW3281	0	0	0	0	0	0	0
UTI89_C3775	b3320	rplC	JW3282	0	0	0	0	0	0	0
UTI89_C3776	b3321	rpsJ	JW3283	0	0	0	0	0	0	0
UTI89_C3803	b3973	birA	JW3941	2	1	2	3	3	5	4
UTI89_C3804	b3972	murB	JW3940	0	0	1	0	0	0	0
UTI89_C3831	b3988	rpoC	JW3951	0	0	0	0	0	0	0
UTI89_C3832	b3987	rpoB	JW3950	0	0	0	0	0	0	0

UTI89_C3834	b3986	rplL	JW3949	0	0	0	0	0	0	0
UTI89_C3835	b3985	rplJ	JW3948	0	0	0	0	0	0	0
UTI89_C3839	b3982	nusG	JW3945	0	0	0	0	0	0	0
UTI89_C3840	b3981	secE	JW3944	0	0	0	0	0	0	0
UTI89_C3842	b3340	fusA	JW3302	0	0	0	0	0	0	0
UTI89_C3843	b3341	rpsG	JW3303	0	0	0	0	0	0	0
UTI89_C3844	b3342	rpsL	JW3304	0	0	0	0	0	0	0
UTI89_C3882	b3384	trpS	JW3347	0	0	0	0	0	0	0
UTI89_C3896	b3398	yrfF	JW3361	0	2	2	0	2	3	2
UTI89_C3942	b3433	asd	JW3396	5	0	1	1	0	0	2
UTI89_C3976	b3461	rpoH	JW3426	0	0	0	0	0	0	0
UTI89_C3977	b3462	ftsX	JW3427	0	0	0	0	0	0	1
UTI89_C3978	b3463	ftsE	JW3428	5	0	7	0	0	1	2
UTI89_C3979	b3464	ftsY	JW3429	0	0	0	0	0	0	0
UTI89_C3987	b3471	yhhQ	JW3436	21	7	28	3	7	14	19
UTI89_C4064	b3532	bcsB	JW3500	100	36	111	28	24	55	68
UTI89_C4100	b3560	glyQ	JW3531	0	0	0	0	0	0	0
UTI89_C4149	b3608	gpsA	JW3583	0	0	0	0	0	0	0
UTI89_C4176	b3633	kdtA	JW3608	0	0	0	0	0	0	0
UTI89_C4177	b3634	coaD	JW3609	0	0	0	0	0	0	0
UTI89_C4181	b3637	rpmB	JW3612	0	0	0	0	0	0	0
UTI89_C4183	b3639	dfp	JW5642	0	0	0	0	0	0	0
UTI89_C4184	b3640	dut	JW3615	0	0	0	0	0	0	0
UTI89_C4193	b3648	gmk	JW3623	4	2	4	0	0	4	0
UTI89_C4195	b3650	spoT	JW3625	9	4	23	1	4	9	7
UTI89_C4249	b3699	gyrB	JW5625	0	0	1	0	0	0	0
UTI89_C4251	b3701	dnaN	JW3678	0	0	0	0	0	0	0
UTI89_C4252	b3702	dnaA	JW3679	0	0	0	0	0	0	0
UTI89_C4254	b3703	rpmH	JW3680	0	0	0	0	0	0	0
UTI89_C4255	b3704	rnpA	JW3681	0	0	0	0	0	0	0
UTI89_C4258	b3705	yidC	JW3683	0	0	0	0	0	0	0
UTI89_C4262	b3709	tnaB	JW5622	204	96	381	67	71	219	232
UTI89_C4281	b3729	glmS	JW3707	0	0	0	0	0	0	0
UTI89_C4282	b3730	glmU	JW3708	0	0	0	0	0	0	0
UTI89_C4351	b3793	wzyE	JW3769	12	1	13	3	9	11	5
UTI89_C4363	b3804	hemD	JW3776	0	0	0	0	0	0	0
UTI89_C4364	b3805	hemC	JW5932	0	1	1	0	0	1	1
UTI89_C4421	b3834	yigP	JW3811	0	0	0	0	0	0	0
UTI89_C4422	b3835	ubiB	JW3812	0	0	0	0	0	0	0
UTI89_C4428	b3843	ubiD	JW3819	0	0	0	0	0	0	0
UTI89_C4435	b3850	hemG	JW3827	0	0	0	0	0	0	0
UTI89_C4454	b3865	yihA	JW5930	0	0	0	0	0	0	0
UTI89_C4518	b3933	ftsN	JW3904	15	7	22	5	6	11	23
UTI89_C4562	b3967	murI	JW5550	0	0	0	0	0	0	0
UTI89_C4610	b4040	ubiA	JW4000	0	0	0	0	0	0	0
UTI89_C4611	b4041	plsB	JW4001	1	0	0	0	0	0	0
UTI89_C4613	b4043	lexA	JW4003	0	0	0	0	0	0	0

UTI89_C4625	b4052	dnaB	JW4012	0	0	0	0	0	0	0
UTI89_C4645	b4059	ssb	JW4020	0	0	0	0	0	0	0
UTI89_C4680	b4084	alsK	JW5724	44	9	62	9	6	36	42
UTI89_C4740	b4142	groS	JW4102	0	0	0	0	0	0	0
UTI89_C4760	b4160	psd	JW4121	5	0	1	1	2	5	1
UTI89_C4762	b4162	orn	JW5740	0	0	0	0	0	0	0
UTI89_C4768	b4168	yjeE	JW4126	0	0	0	0	0	0	0
UTI89_C4802	b4202	rpsR	JW4160	0	0	0	0	0	0	0
UTI89_C4831	b4226	ppa	JW4185	0	0	0	0	0	0	0
UTI89_C4864	b4258	valS	JW4215	0	0	0	0	0	0	0
UTI89_C4868	b4261	yjgP	JW4218	0	0	0	0	0	0	0
UTI89_C4869	b4262	yjgQ	JW5760	0	0	0	0	0	0	0
UTI89_C5067	b4361	dnaC	JW4325	0	0	0	0	0	0	0

B.2 *E. coli* strain EC958



Number of reads obtained for essential genes in EC958. Essential genes plotted if they contained at least one read in any of the 7 TraDIS libraries sequenced. See table overleaf for all orthologs including those with 0 reads in all libraries.

Appendix B

UTI89 Gene	EC958 Gene	Mutant library	37°C biofilm	37°C planktonic	37°C static	25°C biofilm	25°C planktonic	25°C static
UTI89_C0029	lspA	0	0	0	0	0	0	0
UTI89_C0031	ispH	0	0	0	0	0	0	0
UTI89_C0093	ftsI	0	0	0	0	0	0	0
UTI89_C0094	murE	0	0	0	0	0	0	0
UTI89_C0095	murF	0	0	0	0	0	0	0
UTI89_C0096	mraY	0	0	0	0	0	0	0
UTI89_C0097	murD	0	0	0	0	0	0	1
UTI89_C0102	ftsQ	0	1	0	0	0	0	0
UTI89_C0103	ftsA	0	0	0	0	0	0	0
UTI89_C0104	ftsZ	0	0	0	0	0	0	0
UTI89_C0111	coaE	0	0	0	0	0	0	0
UTI89_C0129	lpd	0	0	0	0	0	0	0
UTI89_C0139	can	0	0	0	0	0	0	0
UTI89_C0170	hemL	0	0	0	0	0	0	0
UTI89_C0180	dapD	0	0	0	0	0	1	0
UTI89_C0185	tsf	0	0	0	0	0	0	0
UTI89_C0186	pyrH	0	0	0	0	0	0	0
UTI89_C0187	frr	0	0	0	0	0	0	0
UTI89_C0192	bamA	0	0	0	0	0	0	0
UTI89_C0194	lpxD	0	0	0	0	0	0	1
UTI89_C0196	lpxA	0	0	0	0	0	0	0
UTI89_C0197	lpxB	0	0	0	0	0	0	0
UTI89_C0199	dnaE	1	0	0	0	0	0	0
UTI89_C0200	accA	0	0	0	0	0	0	0
UTI89_C0436	ribD	0	0	0	0	0	0	0
UTI89_C0437	ribE	0	0	0	0	0	0	0
UTI89_C0439	nusB	0	0	0	0	0	0	0
UTI89_C0443	dxs	0	0	0	0	0	0	0
UTI89_C0444	ispA	0	0	0	0	0	0	0
UTI89_C0497	dnaX	0	0	0	0	0	0	0
UTI89_C0503	hemH	0	0	0	0	0	0	0
UTI89_C0555	cysS	0	0	0	0	0	0	0
UTI89_C0636	mrdB	0	0	0	0	0	0	0
UTI89_C0637	mrdA	0	0	0	0	0	0	1
UTI89_C0655	lnt	0	0	0	0	0	0	0
UTI89_C0657	ybeY	0	0	0	0	0	0	0
UTI89_C0982	rpsA	0	0	0	0	0	0	0
UTI89_C0985	msbA	0	0	0	0	0	0	1
UTI89_C0986	lpxK	0	0	0	0	0	0	0
UTI89_C0994	mukF	0	0	0	0	0	0	0
UTI89_C0996	mukB	0	0	0	0	0	0	0
UTI89_C1002	asnS	0	0	0	0	0	0	0
UTI89_C1019	fabA	0	0	0	0	0	0	0
UTI89_C1036	yccK	0	0	0	0	0	0	0

UTI89_C1194	murJ	0	0	0	0	0	0	0
UTI89_C1217	fabD	0	0	0	0	0	0	0
UTI89_C1218	fabG	0	0	0	0	0	0	0
UTI89_C1220	acpP	0	0	0	0	0	0	0
UTI89_C1225	tmk	0	0	0	0	0	0	0
UTI89_C1226	holB	0	0	0	0	0	0	0
UTI89_C1244	lolC	0	0	0	0	0	0	0
UTI89_C1260	purB	0	0	0	0	0	0	0
UTI89_C1398	pth	1	0	0	0	0	0	0
UTI89_C1405	prfA	0	0	0	0	0	0	0
UTI89_C1409	kdsA	0	0	0	0	0	0	0
UTI89_C1551	yciM	0	0	0	0	0	0	0
UTI89_C1561	fabI	1	0	0	0	0	0	0
UTI89_C1714	yneN	0	0	0	0	0	2	0
UTI89_C1853	ribC	0	0	0	0	0	0	0
UTI89_C1906	pheT	0	0	0	0	0	0	0
UTI89_C1934	nadE	0	0	0	0	0	0	0
UTI89_C2001	yeaZ	0	0	0	0	0	0	0
UTI89_C2051	yebG	0	0	0	0	0	0	0
UTI89_C2059	lpxM	0	0	0	0	0	0	0
UTI89_C2070	aspS	0	0	1	0	0	0	0
UTI89_C2462	rplY	0	0	0	0	0	0	0
UTI89_C2512	gyrA	0	0	0	0	0	0	0
UTI89_C2515	nrdA	0	0	0	0	0	0	0
UTI89_C2516	nrdB	0	0	0	0	0	0	0
UTI89_C2601	accD	0	0	0	0	0	0	0
UTI89_C2608	fabB	0	0	1	0	0	0	0
UTI89_C2731	gltX	0	0	0	0	0	0	0
UTI89_C2743	ligA	0	0	0	0	0	1	0
UTI89_C2798	dapE	0	0	0	0	0	0	0
UTI89_C2835	hisS	0	0	0	0	0	0	0
UTI89_C2836	ispG	0	0	0	0	0	0	0
UTI89_C2851	iscU	0	0	0	0	0	0	0
UTI89_C2879	tadA	0	0	0	0	0	0	0
UTI89_C2939	rplS	0	0	0	0	0	0	0
UTI89_C3117	ispF	0	0	0	0	0	0	0
UTI89_C3119	ftsB	0	0	0	0	0	0	0
UTI89_C3149	pyrG	0	0	0	0	0	0	0
UTI89_C3229	thyA	0	0	0	0	0	0	0
UTI89_C3338	yqgF	0	0	0	0	0	0	0
UTI89_C3444	plsC	0	0	0	0	0	0	0
UTI89_C3446	parC	0	0	0	0	0	0	0
UTI89_C3454	3424	14	5	35	6	8	24	15
UTI89_C3598	infB	0	0	0	0	0	0	0
UTI89_C3608	glmM	0	0	0	0	0	0	0
UTI89_C3610	ftsH	0	0	0	0	0	0	0
UTI89_C3618	rpmA	0	0	0	0	0	0	0

UTI89_C3620	rplU	0	0	0	0	0	0	0
UTI89_C3621	ispB	0	0	0	0	0	0	0
UTI89_C3637	lptB	0	0	0	0	0	0	0
UTI89_C3689	accC	0	0	0	0	0	0	0
UTI89_C3732	fmt	1	0	1	0	0	1	1
UTI89_C3739	rplQ	0	0	0	0	0	0	0
UTI89_C3747	rplO	0	0	0	0	0	0	0
UTI89_C3748	rpmD	0	0	0	0	0	0	0
UTI89_C3750	rpsE	0	0	0	0	0	0	0
UTI89_C3751	rplR	0	0	0	0	0	0	0
UTI89_C3753	rplF	0	0	0	0	0	0	0
UTI89_C3755	rpsH	0	0	0	0	0	0	0
UTI89_C3756	rpsN	0	0	0	0	0	0	0
UTI89_C3757	rplE	0	0	0	0	0	0	0
UTI89_C3758	rplX	0	0	0	0	0	0	0
UTI89_C3760	rplN	0	0	0	0	0	0	0
UTI89_C3761	rpsQ	0	0	0	0	0	0	0
UTI89_C3762	rpmC	0	0	0	0	0	0	0
UTI89_C3764	rplP	0	0	0	0	0	0	0
UTI89_C3765	rpsC	0	0	0	0	0	0	0
UTI89_C3767	rplV	0	0	0	0	0	0	0
UTI89_C3768	rpsS	0	0	0	0	0	0	0
UTI89_C3770	rplB	0	0	0	0	0	0	0
UTI89_C3771	rplW	0	0	0	0	0	0	0
UTI89_C3773	rplD	0	0	0	0	0	0	0
UTI89_C3775	rplC	0	0	0	0	0	0	0
UTI89_C3776	rpsJ	0	0	0	0	0	0	0
UTI89_C3843	rpsG	0	0	0	0	0	0	0
UTI89_C3844	rpsL	0	0	0	0	0	0	0
UTI89_C3882	trpS	0	0	0	0	0	0	0
UTI89_C3896	yrfF	0	2	2	0	2	3	2
UTI89_C3979	ftsY	0	0	0	0	0	0	0
UTI89_C4099	glyS	0	0	0	0	0	0	0
UTI89_C4100	glyQ	0	0	0	0	0	0	0
UTI89_C4164	rfaD	0	0	0	0	0	0	0
UTI89_C4166	rfaC	0	0	0	0	0	0	0
UTI89_C4181	rpmB	0	0	0	0	0	0	0
UTI89_C4184	dut	0	0	0	0	0	0	0
UTI89_C4251	dnaN	0	0	0	0	0	0	0
UTI89_C4252	dnaA	0	0	0	0	0	0	0
UTI89_C4281	glmS	0	0	0	0	0	0	0
UTI89_C4282	glmU	0	0	0	0	0	0	0
UTI89_C4422	ubiB	0	0	0	0	0	0	0
UTI89_C4428	ubiD	0	0	0	0	0	0	0
UTI89_C4613	lexA	0	0	0	0	0	0	0
UTI89_C4645	ssb	0	0	0	0	0	0	0
UTI89_C4740	groS	0	0	0	0	0	0	0

UTI89_C4741	groL	0	0	0	0	0	0	0
UTI89_C4760	psd	5	0	1	1	2	5	1
UTI89_C4831	ppa	0	0	0	0	0	0	0
UTI89_C4864	valS	0	0	0	0	0	0	0
UTI89_C5081	holD	0	0	0	0	0	0	0

Appendix C **Publications**

Data from this thesis has not yet been published. However, Shannon Fenlon has contributed to the following papers over the course this PhD.

1. Dikshit N, Bist P, **Fenlon SN**, Pulloor NK, Chua CE, et al. (2015) Intracellular Uropathogenic E. coli Exploits Host Rab35 for Iron Acquisition and Survival within Urinary Bladder Cells. PLoS Pathog 11: e1005083.

2. Ivan Sovic, Mile Sikic, Andreas Wilm, **Shannon Nicole Fenlon**, Swaine Chen, Niranjan Nagarajan (2016) Fast and sensitive mapping of nanopore sequencing reads with GraphMap. Nat Commun 7:11307