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Functional Analysis of Fic Domain Bearing Proteins in *Klebsiella pneumoniae.*

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AL MADADHA, MOHAMMAD E. (DR.), III DEPARTMENT.

Statement of originality

This accompanying thesis submitted for the degree of PhD entitled:

"**Functional Analysis of Fic Domain Bearing Proteins in *Klebsiella pneumoniae***" is based on work conducted by the author at the University of Leicester mainly during the period between 5th April 2013 and 31st March 2016.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of the work has been submitted for another degree in this or any other university.

Signed

Date:

Abstract

Fic domain bearing proteins are characterized with the presence of a domain carrying the conserved amino acid sequence (HPFX(D/E)GNGR). These proteins are present in all walks of life but are more concentrated within prokaryotes and more so within bacteria. In recent years these proteins have been increasingly characterized and their role in bacterial virulence is being elucidated. Fic proteins are usually secreted by bacteria and once delivered into the host cell they usually target cytoskeleton regulating G proteins that affect the target host in different ways depending on the target G protein. In *Klebsiella pneumoniae,* we have identified 5 homologous genes that code for five different Fic domain bearing proteins in this bacteria. Our first effort was to determine if these proteins were secreted, to verify if they adhere to the toxin-secretion system paradigm. The work then focused on determining the virulence effect of these proteins in an in vivo assay, an in vitro assay and an enzymatic assay for the Fic-RL protein. Fic-RL is one of the five proteins in *K. pneumoniae* have been shown to be conserved in all 80 sequenced strains of this bacteria. In our secretion assay this protein have been shown to be secreted by the bacteria, moreover, by utilizing different mutants that lacked different parts of secretion systems, we have been able to determine that the T4SS present on the Integrative and Conjugative Element 1 (*icekp1*) to be responsible for the secretion of this protein and not the other 4 homologs. Virulence assays showed that when this protein was expressed with eukaryotic cells by means of transfection, confocal and fluorescent microscopy revealed that cytotoxic effect are evident as cell rounding and actin cytoskeletal collapse in transfected cells, which does not occur when a mutated version of the protein is instead expressed. Survival killing assays utilizing the larvae of the *Galleria mellonella* wax moth, revealed significant attenuation of strains lacking the gene coding for Fic domain bearing proteins, which is partially complemented by re-introducing the genes in plasmid constructs. The enzymatic function for the Fic-RL protein was shown in a Guanine Exchange Factor assay, designed to reveal if a protein is able to utilize fluorophore bound substrates in its reaction of provided G protein targets (Cdc42, Rac1, RhoA), showed that Fic-RL is able to cause an increase in relative fluorescence measured that was similar to a known Guanine Exchange Factor (hDbs) but only when used on Cdc42 and not the other two G proteins.

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Common Abbreviations

ATP: Adenosine Triphosphate

AMP: Adenosine Monophosphate

BLAST: Basic Local Alignment Search Tool

Bp/bp: Base Pair

dNTP: deoxyribonucleoside triphosphate mix

DNA: deoxyribose nucleic acid

FIC: Filamentation Induced by Cyclic-AMP

GEF: Guanine Exchange Factor

IPTG: isopropyl-β-D-thiogalactopyranoside

Kd: Kilo Dalton

LA: Luria Bertani agar

LB: Luria Bertani broth

MCS: Multiple Cloning Site

mM: milliMolar

M: Molar

NCBI: national centre for biotechnology information

OD: Optical Density (e.g OD600: Optical Density at absorbance of 600nm)

ORF: Open reading frame

PCR: Polymerase Chain Reaction

PBS: Phosphate buffered saline

PTM: Post Translational Modification

RPM: Rotation Per Minute

SDS-PAGE: Sodium Dodecyl Sulfate PolyacrylAmide Gel Electrophoresis

T3SS Type 3 Secretion System

T4SS Type 4 Secretion System

T2SS Type 2 Secretion System

WB: Western Blot

^{o} C: Degree Celsius

λ: Lambda

µl: Microlitre

µg: Microgram
µM: MicroMolar

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Chapter 1

Introduction

1.1 An Overview of *Klebsiella* Species

The genus *Klebsiella* of the family *Enterobacteriaceae* is characterised as non-motile, encapsulated rod-shaped bacteria with a negative reaction to the Gram stain. The *Klebsiella* species are commonly encountered on the surface of mammalian mucosa, including humans, and in the environment on aquatic surfaces, foliage, and soil. Bacteria isolated from the two sources are highly similar in terms of virulence and biochemical characteristics[1].

The species that are most commonly encountered in human host are *Klebsiella pneumoniae* and much less commonly *Klebsiella oxytoca[2]*.

1.1.1 Identification of *Klebsiella* Species

Klebsiella genus of bacteria belongs to the family *Enterobacteriaceae. Klebsiella* is usually identified by its lack of oxidase enzyme, harbouring catalase enzyme, utilisation of citrate, being a nitrate reducer and its ability as a facultative anaerobic glucose fermenter (the classical fermentation of lactose on MacConkey agar gives rise to pink colonies on the red coloured agar). Other tests are also usually utilised to distinguish it from other close members of the *Enterobacteriaceae* family (such as *Escherichia coli* and *Enterobacter* species) such as Voges-Proskauer test and Methyl Red test[3].

1.1.2 *Klebsiella pneumoniae* as a Human Pathogen

K. pneumoniae, the most impactful subspecies of the *Klebsiella* family on human health, is a cause for a multitude of infections in various tissue types that ranges from lungs, blood, urinary tract, meninges and even skin and soft tissue infection. The usual common predisposing factor of these infections is the presence of a chronic illness or an immune compromised state[2].

In the community, the bacteria are often encountered clinically as a cause of community-acquired pneumonia especially among populace affected by chronic alcoholism, and to lesser degree as a cause of urinary tract infection (UTI); however, this profile drastically changes in the hospital setting where *K. pneumoniae* is a cause of serious and sometimes fatal infections[4].

This can be attributed to the carriage state of *K. pneumoniae,* where in the community it is usually found as part of the normal flora in the gastrointestinal tract and nasopharynx at rates that reportedly range from 5-38% and 1-6%, respectively. However, in the hospital setting the rates are much higher and are reported to be as high as 77% in stool samples, 19% in the nasopharynx and 42% on the skin of the hands[2].

The main identified factors that are associated with the increased carriage rate or resultant infection caused in nosocomial settings are [2, 5]:

- Previous hospital stay
- Previous exposure to antibiotics
- Length of hospital stay
- Underlying illnesses (chronic obstructive pulmonary disease, diabetes mellitus, immune compromised state...)
- Catheters and other similar mechanical devices

These factors contribute to this opportunistic pathogen's ability to form a continuous reservoir of infection that spreads among patients in the hospital setting and cause a host of serious conditions that ranges from UTI, pneumonia, and septicemia to even ,in hyper virulent strains, can cause gall bladder infection, pyogenic liver abscess formation and wound infections[6]. The ability to infect different tissue types signify the pathogenicity of *Klebsiella* spp. and their ability to thrive under variable host conditions[7].

Hypervirulent *K. pneumoniae* strain are the primary cause of serious lung and liver abscess formation in eastern Asian countries but can cause other infections as well [8], these hypervirulent strains of *K. pneumoniae* acquire genes coding for virulence factors that enhance its ability to cause infection or evade the host's defenses[9]. The concentration of these virulence factors make *K. pneumoniae* a more serious pathogen to deal with, not only in the hospital setting, but these hypervirulent strains are being increasingly seen in community acquired infections as well[10]. The danger of these bacteria is further elevated when some strains acquire resistance to most (or even all) antibiotics that can combat this bacterium. Leaving us with the fact that the body's defenses is unable to deal with hypervirulent strains (or even classical strains in immunocompromised patients) and having few or no options for treating these infections with our current antibiotic arsenal (see below). Although strains are usually harboring antibiotic resistance genes or are hypervirulent strains acquiring more virulence genes, there are recent reports that confirm the evolution of hypervirulent strains that are also resistant to antibiotics [11].

1.2 Pathogenicity and Virulence Factors of *Klebsiella*

The factors that make *Klebsiella* species virulent or contribute to its virulence are described below, each pathogenicity factor is described according to the host defense mechanism that it counteracts.

1.2.1 Capsular Polysaccharides (CPS)

The classical capsule that is produced by these bacteria is one if its best defense mechanisms against phagocytosis by host immune cells. The capsule prevents opsonisation by host immune cells which would target them for phagocytosis by macrophages[12]. The resultant decrease in the uptake of these bacteria by immune cells thwarts and suppresses the usual intensity of the immune system against such an invading host, resulting in an overall decrease in pro-inflammatory cytokines (and higher

level of anti-inflammatory cytokines) as seen in capsulated strains vs non-capsulated strains[13].

Strains that do not produce a capsule are significantly less virulent as measured by mouse mortality rates and bacterial load of capsule Vs un capsulated strains[14] while strains that produce a hypercapsule (as seen in hypervirulent strains) show enhanced resistance to humoral mediated immune responses[15].

The CPS are the building blocks of this hallmark capsule, which gives *Klebsiella* colonies their distinct mucoid appearance. CPS is made up of repeated units of 4-6 heteropolysaccharides, it is with these subunits that *Klebsiella* is often classified into different serological subtypes, of the many different capsule antigens; 77 CPS types have been widely used for serotype recognition[2].

The capsule is formed by the tight clustering of CPS subunits into large, dense fibrillous architecture around the surface of the bacterium. This capsule serves as a barrier against the host's bactericidal elements present in the serum, and also prevents phagocytes from enveloping the bacterium by inhibiting opsonization of complement factors to the surface of the cell [16].

Out of the many known capsule serotypes, capsule types K1, K2, K4 and K5 are reportedly more virulent than others. On closer inspection of the serotypes' structure, it appears that less virulent serotypes contain significantly more repeating sequences of a specific mannose or rhamnose sugar. These sugar bases are recognised by a surface lectin present as a receptor on macrophages and initiate an opsonin-independent phagocytosis termed as lectinophagocytosis. Indeed, the more virulent serotypes, such as K2, lack these sugar moieties in their capsule structure [17, 18].

1.2.2 Fimbriae (pili) and biofilm formation

For *Klebsiella* to be successful at causing infection, it must traverse the trials imposed on it by the host's defenses. The very first mechanism that the host (the human body in this case) combats invading microbes is through mechanical removal of these organisms. Urine flow in the urinary tract or the ciliated movement of mucous outside the respiratory tract or the peristaltic movement of the bowels establishes mechanical removal of most invading microbes and more importantly prevents them from binding to the tissues. For a bacterial cell to overcome this trail, a close proximity must be maintained with the target tissue. In *Klebsiella,* this is achieved by the fimbriae structures. These structures project outwards from the outer cell membrane, through the capsule, to come in contact and cling to specific targets at the host's mucosal surface [19, 20].

Two main fimbriae are described: type 1 fimbria which is shared among all members of the *Enterobacteriaceae* family. They owe their function to cling to the mucosa to the FimH adhesin part present at the tip of the structure of this organelle. It is able to bind to certain glycoproteins with mannose moieties present on multiple mucosal surfaces such as the epithelium of the urinary tract[20].

The type 3 fimbria, although smaller in size, is like the first type in that it has a main subunit (MrkA) protruding from the cell, and a tip based adhesin unit (MrkD) recognising different tissue types than type 1 fimbriae, allowing it to attach and consequently infect different tissues. The MrkD adhesin recognises basement membrane and tracheal surface components instead [19].

Biofilm formation can be thought of as an extended mechanism for the resistance of bacteria (including *Klebsiella*) against mechanical removal by the body's defenses. In a biofilm bacteria settles in niches and surround their entire colonies by producing a thick layer of proteins and polysaccharides, which helps these colonies stick to surfaces of tissues [21]. Not only does this thick layer of slime prevent these bacteria from mechanical removal, it also shields them from body immune response as well as

prevents antimicrobial agents from reaching the bacteria within a biofilm, where it has been shown that bacteria within biofilms are usually more resistant to antibiotics[22]. Fimbriae as well as CPS both improve the production of biofilm formation, both of which are virulence factors that Klebsiella is known to have[23].

1.2.3 Complement Evasion and Lipopolysaccharides (LPS)

Apart from phagocytosis and cell mediated immune response, the body can mount a humoral immune response, like the activation of the complement pathways. the complement system that is ultimately able to induce a bactericidal effect upon its activation by producing membrane attack complexes which bind to the surface of cells targeted by this system and bore holes within its membrane that leads to its lysis of the targeted cell. Complement pathways also induce the production of pro inflammatory cytokines that recruit more immune cells and mediators, and lastly the complement system also flag cells for phagocytosis by means of opsonins [24, 25].

The complement system depends on the activation of the C5b-C9 cascade that mediates the cell killing activity through destabilising the osmotic balance of the target cell by inducing multiple pores that span the depth of the bacterium's membrane.

This cascade can be activated through three different routes: the classic antibody-mediated pathway is activated by complement binding to antibody-antigen complexes. Second is the alternative pathway which is less specific route independent of antibodies and is regarded as a first response defense mechanism to allow the host to defend against an invading pathogen until more specific antibodies provide a more reliable response and finally the lectin pathways. The alternative pathway is activated by spontaneous conversion of the C3 molecule into C3b, C3b in turn flags bacteria that lacks sialic acid on its surface [17]. Lastly is the lectin pathways, which is like the classical pathway but differs on the fact that it employs mannose- binding lectins in its pathway activation rather than C1q, the mannose binding lectin identify mannose moieties on bacterial surfaces for complement activation[26].

These pathways lead to the activation of complement factor C3, which in turn forms the opsonin C3b that is responsible for the induction of the C5b-C9 cascade.

LPS is an integral part of the Gram-negative cell's outer membrane and is a major target of the host immune response. The ability of an invading pathogen to escape the killing effect of the serum complement components is vital for survival and maintenance of infection [27]. LPS is made up of O antigen (which is used also for serotyping of which 9 serotypes for *K. pneumoniae* is identified thus far), oligosaccharides and lipid A. the structure of the LPS is a major activator for immune response through complement pathway, where *K. pneumoniae* activates all three pathways when its LPS is recognized by the immune cells.

Pathogenic strains of *Klebsiella*, as opposed to non-pathogenic strains, can resist serum defenses by unknown mechanisms. One hypothesis involves the capsule's ability to hinder C3b attachment, others involve the smooth structure of the LPS that seems to only activate the classic complement pathway, and not the alternative pathways, giving the bacterium some leeway to establish an infection before the host is able to mount a response. New evidence points to the contribution of two of the outer membrane proteins, the murein lipoprotein(Lpp) and the peptidoglycan-associated lipoprotein (Pal), it has been reported that mutants lacking these two proteins were more readily eliminated when exposed to human sera than the wild-type strain, suggesting a role of these proteins in serum defense.[28]

1.2.4 Siderophores

Iron trapping or chelating is vital for the energy production in bacteria as iron is an integral part of the electron transport activity. In the host environment, free iron concentrations are far too low to sustain bacterial growth, let alone to thrive as an infecting agent. Iron is supplied by iron chelating proteins called siderophores that competitively trap iron that is bound to iron binding serum proteins such as transferrin and lactoferrin [29], making siderophores essential for *Klebsiella* virulence[1, 30]. In

human host, certain molecules are secreted that counter act siderophores' ability to trap iron (such as lipocalin-2). However, in *K. pneumoniae* more than one type of siderophore is utilized, and some of those types are not counter acted by lipocalin-2. Enterobactin is the classical or default siderophore present in all strains, and is the type of siderophore that is inhibited by lipocalin-2. More virulent types of K. pneumoniae acquire other types of siderophores, such as salmochelin (glycosylated form of enterobactin), yersiniabactin and aerobactin are two distinct forms of siderophores from enterobactin, however yersiniabactin activity is slightly hindered by transferrin in humans. More aggressive strains of *K. pneumoniae* utilize different forms of these siderophores that allow them to survive in different niches in their host[6, 29-32].

1.3 The Threat of *Klebsiella*

As described above *Klebsiella* is a competent opportunistic pathogen with its many virulence factors. However, this competency of causing infection is further augmented by the fact that some *Klebsiella* strains that have acquired resistance to a wide array of antibiotics. Some of those antibiotics such as carbapenem and colistin have been deemed as "last line of defence" antibiotics [5, 33].

Recently the Center for Disease Control has published their annual report depicting the real threat of Klebsiella as the overwhelmingly dominant causative agent of infections caused by Carbapenem-resistant *Enterobacteriaceae* (CRE)[34].

The CDC reports resistance to beta-lactam antibiotics, the genera of drugs usually used for treating Gram-negative infections, has reached levels of high urgency. Resistance to narrow spectrum older generation beta lactams commonly seen among members of Gram-negative bacteria, especially *Enterobacteriaceae,* and resistance to newer extended spectrum beta lactams (cephalosporins) spreading beyond the hospital setting to be seen among community-acquired strains. *K. pneumoniae* has been shown

to possess the enzyme that enables it to confer resistance to these beta lactam antibiotics (ESBL enzyme).

Last resort drugs such as carbapenems and polymixins have also been added to the list of drugs these bacteria have acquired resistance to, giving credibility to the reports claiming of nearing a post-antibiotics era. This, coupled with the toxicity of some of the last resort drugs and the toxicity resulting from combination therapy (one of the methods used to battle CRE infections) only adds to the morbidity of these infections.

In the United States alone, nearly 9000 CRE annual infections have been reported with a death toll close to 600 per annum, which have placed CRE as one of three groups of organisms with the highest threat level *Klebsiella* reserves the lion's share of these numbers. Similar or even higher numbers of morbidity and mortality are being reported all over the world [5, 33, 35].

1.4 The Fic Domain

1.4.1 Overview

Fic proteins are a family of proteins that bear the domain labelled as FIC (which stands for **F**ilamentation **I**nduced by **C**yclic AMP). The Fic domain was first characterised in *E. coli* causing the induction of Filamentation of this bacteria upon the addition of Cyclic AMP (Filamentation Induced by Cyclic AMP or FIC) [36]. Fic family of proteins are present in many walks of life as shown in figure 1, but they are more abundantly present among bacteria (either pathogenic or non-pathogenic bacteria).

The domain is recognised in proteins by the presence of the amino acid conserved sequence HPFX(D/E)GNGR, which is located within usually a helical shaped domain. The proteins that carry this domain (which has been described in the literature so far) typically are secreted toxins that are secreted through T3SS or T4SS. However, the Fic

family also includes members of toxin-anti toxin proteins where the toxin (a member of the Fic family such as the doc toxin) has its function regulated by an anti-toxin (PHD antitoxin in the case of the doc toxin [37]).

Proteins that fall within these described parameters of structure and function are classified as members of this proteins superfamily. Different bacteria might contain more than one type of Fic domain bearing proteins, and some of those proteins may contain more than one Fic domain. However, the protein is still labelled as a Fic protein or Fic domain bearing protein, despite their varying structural archetypes that are described in the pfam database.

1.4.2 Mechanism of Action and Structure

Although the function for the majority of the Fic proteins remains elusive, the few described Fic proteins have many commonalities that bind them together (Table 1.1) (Figure 1-2). Firstly, every Fic protein performs one form of post-translational modification or another and all the described Fic proteins use a phosphate containing substrate as the means by which they induce a post-translational modification of the target protein [38-44].

The substrates vary from nucleotide monophosphates (AMP or UMP[39, 43]) to Phosphocholine[40, 45] or just a phosphate group as a substrate for phosphorylation[46] and as described in [41] (see below for substrate selection). These phosphate substrates are resourced or obtained by the FIC motif from phosphate-containing molecules (such as ATP), which are catalyzed by the Fic domain to transfer the resulting phosphate containing substrate (e.g. AMP) and transfer it to the target amino acid on the target protein (e.g. Rho G-protein) which would prevent the target protein (Rho GTPase) from exerting its regulatory functions on its downstream effectors.

It has been postulated that the orientation of the binding between the Fic protein and the substrate that provided the phosphate seems to contribute to determining which

of the two possible molecules that contain phosphate is to be transferred to the target protein (for example if ATP is used, the reaction would either use AMP or phosphate as the result of the breaking of the ATP molecule). This post-translational modification usually targets Tyrosine, Threonine or Serine amino acids (all hydroxyl containing amino acids) completing the picture of the substrate and target of the proteins so far [45, 47, 48].

Target and substrate selection:

The relative consistency in the mechanism of action can be illustrated in the crystal structure of Fic proteins that are available so far. As more crystal structures for Fic proteins are being revealed it is shown that a general pattern is revealed. The Fic domain itself is usually within 6 alpha helices that hold within their structure the active site loop containing the FIC motif with the conserved histidine in the motif that is vital to carry out the post-translational modification on the target hydroxyl group [45, 47, 49].

Target selection has been attributed to the more variable regions of the Fic proteins namely the beta hairpin that is within proximity to the active site and serves as an anchor to the target protein (sometimes referred to as a flap). The core Fic domain and its surrounding sites, as well as the flap, all play a role in the selection of the substrate, as substrates seem to usually be AMP, a slight modification of the balance surrounding the active site can relax the substrate specificity and allow the protein to utilize other nucleotides [39, 50-52].

Table 1-1 lists the Fic proteins that have been described in the literature, with an emphasis on target, function and the substrate utilized to carry out the post translational modification.

Fic Protein	Species and reference	Target	Substrate	Phenotype
-IbpA ***-VopS*** **-HYPE** ***-AvrAC***	*-Histophilus somni [53]* *-Vibrio parahaemolyticus [43]* -Humans and animals[39] *-Xanthomonas campestris[54]*	Rho GTPases	Nucleoside monophosphate (AMP or UMP)	Cytoskeletal destabilization or collapse
-AnkX ***-DrrA***	*-Legionella pneumophila[45]* *-Legionella pneumophila[55]*	RAB GTPases	Phosphocholine	Diversion of vesicular transport
Doc Toxin	Many bacterial species[46]	Elongation Factor TU(EF-Tu)	Phosphate (direct phosphorylation)	Stopping ribosomal translation and result in cell death

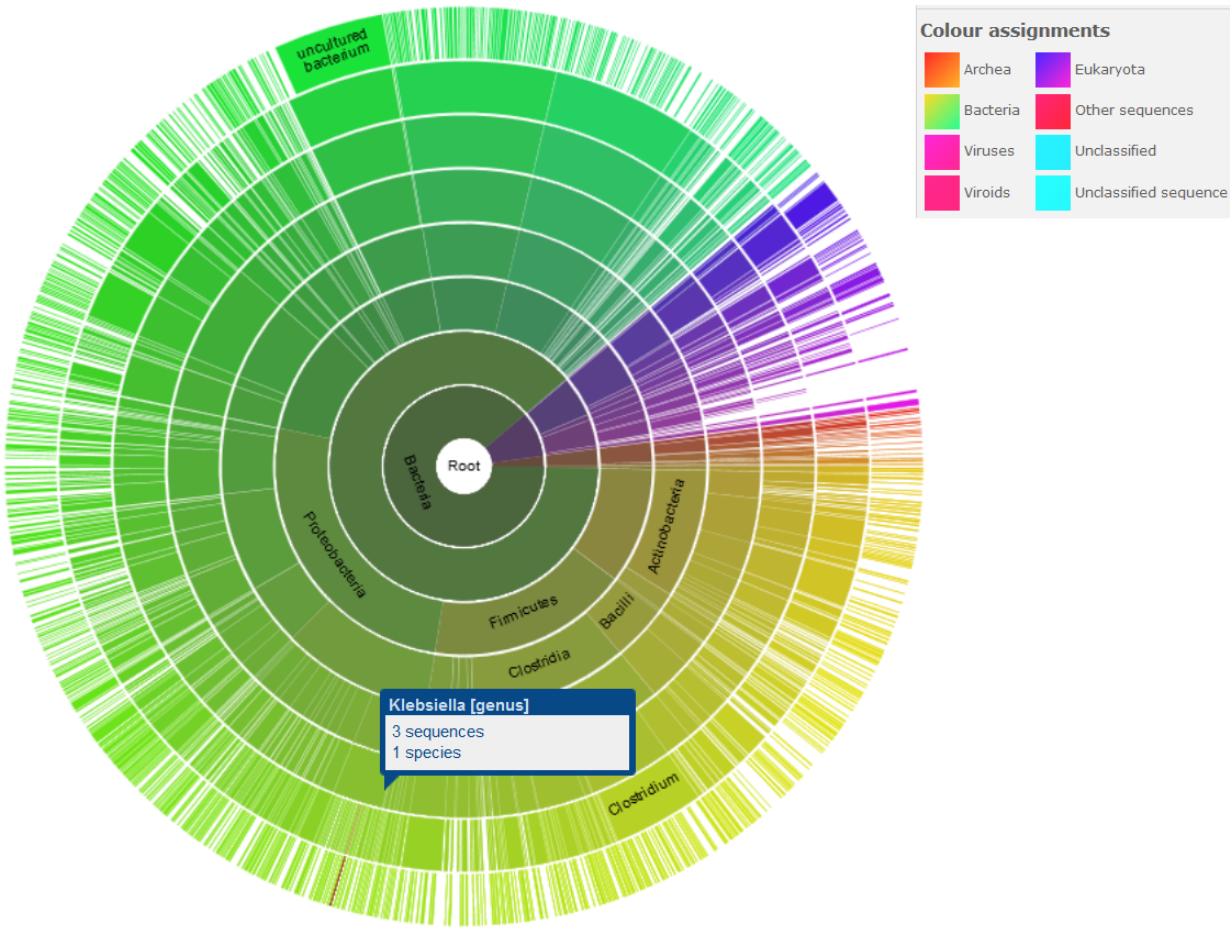


Figure 1-1 distribution of the Fic domain as shown in the pfam database. A search of the presence of this domain was conducted on the protein family database (pfam); following the search a sunburst figure was obtained to show the representation of this domain among all walks of life. The overwhelming majority of known Fic domain bearing proteins (of all proteins entered into the database) are present in bacteria, as shown in the green to yellow spectrum in the figure. Sunburst segments in this figure are weighed by the number of species deposited in the database and not by the number of sequences. Little dark blue arrow box is indicating those Fic proteins sequences identified in *Klebsiella pneumoniae.* Image was produced on the pfam website http://pfam.xfam.org/family/PF02661.16#tabview=tab7 and produced using software provided on the pfam website

1.4.3 Fic Proteins as Virulence Factors

Several Fic domain proteins were found to be important virulence factors, and many of them are toxins secreted by the T3SS[43] and T4SS [49] or in the case of IbpA a toxin that is directly taken up by the host cells.

These proteins once inside the host cell are aimed to subvert the host's defenses and help the invading microorganism to establish an intracellular infection by acting on either RAB or Rho GTPases.

1.4.3.1 Fic protein that Targets RAB GTPases

Legionella pneumophila AnkX protein is the Fic protein so far that has been shown to act on RAB GTPase. AnkX is introduced into the cell by a type 4 secretion system, it functions on RAB GTPase by phosphocholination, which helps establish infection vacuoles by disrupting the actin dependent vesicular trafficking system (controlled by the RAB GTPases). This disruption of the vesicular trafficking system prevents the destruction of internalised bacteria and establishes vacuoles containing the bacteria that ultimately leads to evading the intracellular defences [40, 45, 55].

1.4.3.2 Fic proteins Targeting Rho GTPases

Subversion of Rho GTPases is used by some bacterial pathogens to induce host cell cytostasis and avoid host immune detection. Two examples of this are the VopS (from *Vibrio parahaemolyticus)* and IbpA (from *Histophilus somni*) [39, 43, 56]. These proteins when introduced into the host cell exert their action on Cdc42 or Rac which are major constituents of the Rho GTPase subfamily that controls actin filament structures and functions related to these structures.

VopS was the first described Fic protein that affects Rho GTPases. Yarborough et al [43] were able to determine the mechanism of action of VopS by incubating it with Rac.

Following incubation, Rac showed a molecular weight increase that corresponds to the addition of an AMP (adenosine 5' monophosphate) residue. This residue was specifically attached to threonine 35 of Rac, and the process of addition of AMP was termed AMPylation.

Utilising a mutant form of VopS (where the conserved Histidine in the Fic Motif of VopS was changed to Alanine), further determined consequences of the AMPylation of Rac revealing three key effects that affect the pathway controlled by Rac (and other Rho GTPases) by[43, 47]:

Firstly, eukaryotic cells transfected with VopS showed a disrupted actin cytoskeleton manifested as cell rounding and collapse. This effect was not seen upon transfecting these cells with the mutant form of VopS[43, 47].

Secondly, the activities of the wild-type VoPs but not the mutant caused loss of bidning of Rac to one of its downstream effectors; PAK [43]. A closer look at the function of PAK protein (p21 Activated Kinases) revealed the connection between the observed phenotype of the cytoskeleton when the VopS is introduced into the cell.

Lastly, the mutant VopS (and not the wild-type form) was preferentially binding to GTP-loaded Rac (without reversibility), acting as a substrate trapping molecule to Rac. This led the researchers to believe that the enzymatic activity of VopS depended on the conserved Histidine.

The consequences of the host actin cytoskeleton disruption and the benefit to the invader, have been described for some of the pathogens possessing Rho GTPase altering Fic proteins, such as VopS and IbpA. These proteins affect and hinder the host's innate immune response by altering the ability of phagocytes to engulf that invading bacterium as the actin cytoskeleton regulation is crucial for this mechanism [57-59]

In the case of IbpA, strains that expressed this protein were able to cause the cytoskeletal collapse and cytotoxicity attributed to targeting Rho GTPases in Bovine

Alveolar Type 2 cells (BAT2), whereas strains that lacked this protein were not able to exhibit this phenotype (which was determined in the same study to be caused by adenylation of the Rho GTPases by IbpA)[53]. Moreover, strains that lacked IbpA were not able to migrate between layers of BAT2 cells unless the cells were pretreated with IbpA, added to that antibody produced against IbpA protected the monolayers from bacterial migration through the BAT2 monolayer [51, 53, 60]. This confirmed virulence studies that showed that antibodies to IbpA protected cattle from pneumonia caused by this organism [43, 44, 60].

The important role of IbpA in virulence was shown when two strains of *H. somni* that naturally differ in the presence or absence of the gene encoding for IbpA were compared. The strain that harboured the gene was a virulent pathogen causing bovine pneumonia, whereas the strain that harboured a natural deletion of the gene encoding for IbpA was an asymptomatic strain of *H. somni* [53, 60].

1.4.3.3 Fic Proteins Targeting EF-Tu GTPase

Some of the Fic proteins affect prokaryotes (e.g. bacteria) themselves, as seen in the toxin- anti-toxin (TA) system Doc-Phd. Elongation Factor Tu (EF-Tu) is affected by the Doc toxin halting all cellular transcription in the absence of the anti-toxin Phd [38, 46]. In this example, Fic proteins exert actions similar to those that they utilize to evade host immune systems in order to establish genetic variation regulation and control growth[61]. Bacterial populations that donate genetic material either vertically or horizontally will negatively select other bacteria that does not produce the anti-toxin. Toxin- antitoxin systems are not per se virulence factors, however, their close relationship to virulence factors is evident as shown by crystal structure analysis of the Doc toxin, exhibiting structural similarities to eukaryotic toxins [38, 61].

1.4.3.4 Fic Protein Targets that are not GTPases

The usual pattern of Fic protein target was thought to be as small G protein or GTPases, which are proteins that are activated by exchanging GTP for bound GDP. Fic proteins hijack their mechanism once secreted or taken up by the host to induce an effect that promotes the owner of the Fic domain bearing protein to establish itself as an intracellular pathogen or in case of TA systems establish the strain with the genes encoding for the anti-toxin over other bacteria which lack this system.

However, some targets have been shown to not adhere to this pattern. Type III secreted effector of the bacterium *Xanthomonas campestris* (plant pathogen), AvrAC, has been shown to enhance the virulence by contributing to the evasion of the host's immune response. AvrAC is an uridylyltransferase, acting to induce post-translational modification (also using a phosphate-based molecule, UMP in this case) on plant kinases that are mediators for immune signal transduction [62]. AvrB is another potential effector[39]; however, the target of this protein has not been shown, this T3SS secreted plant protein induces PTM much like Fic proteins do, however, without having a Fic domain (although the topology of this protein has been shown to share similar topology to other Fic proteins)[39, 50, 62, 63]. AvrB protein shares many similarities with Fic proteins (in virulence function and enzymatic activity), it does so without having the Fic motif. However, but it does harbor the backbone that harbors the motif, which shows the complexity of these virulence factors [63].

BepA, another Fic protein secreted by a type IV secretion system was shown to subvert host immune defences. It prevents the affected cell from propagating apoptotic signals by subverting GαS dependent cAMP production. The study did not directly link the Fic domain to this action [49, 64, 65]; however, it provides more insight of how Fic domain virulence factors are aimed at not destroying the cell but help to evade the host's immune defences. This is supported by the evidence that type IV secreted proteins including BepA are essential for the virulence for *Bartonella henselae*[65].

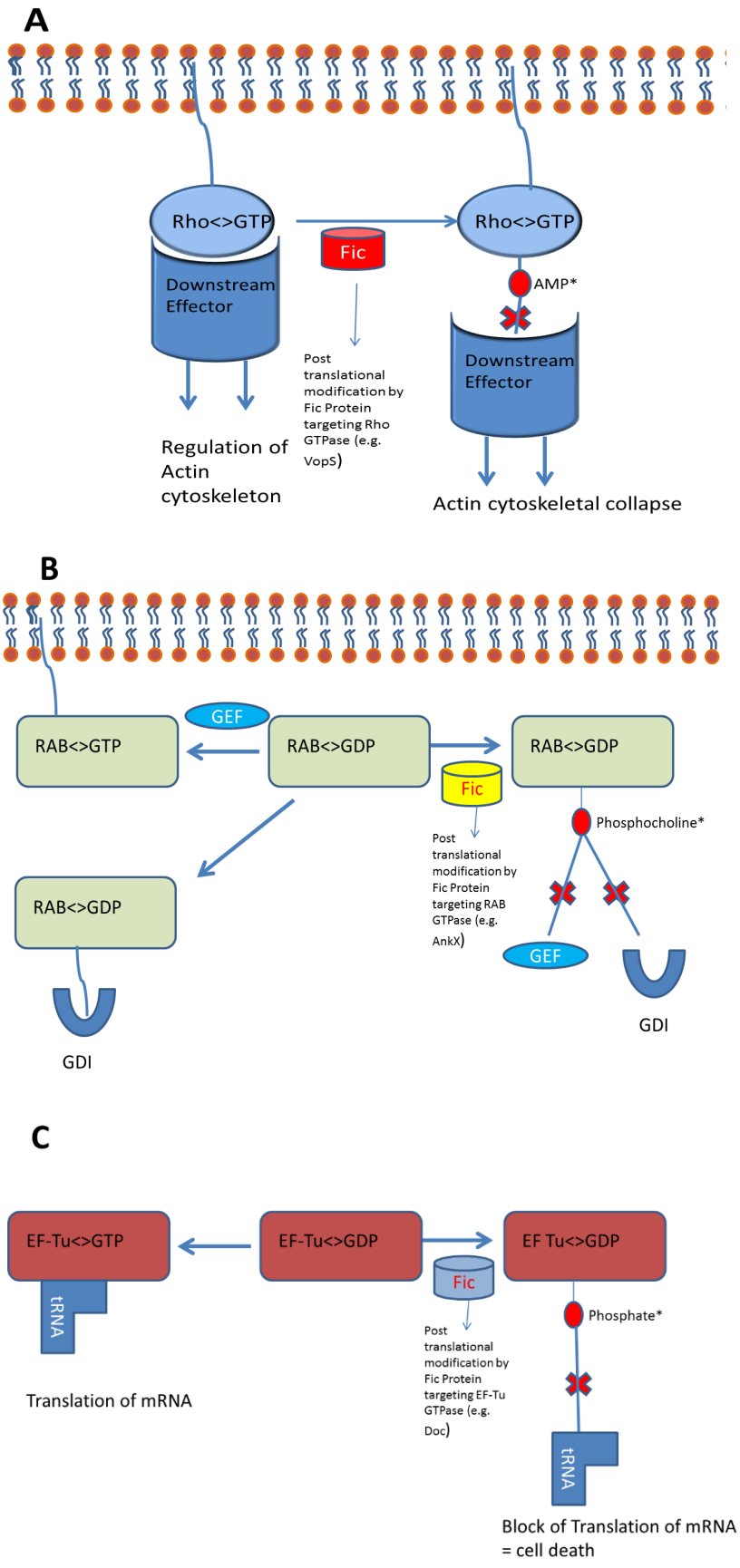

A
Rho<>GTP
Fic
Rho<>GTP
Downstream Effector
AMP*
Downstream Effector
Post translational modification by Fic Protein targeting Rho GTPase (e.g. VopS)
Regulation of Actin cytoskeleton
Actin cytoskeletal collapse
B
GEF
RAB<>GTP
RAB<>GDP
RAB<>GDP
Fic
Phosphocholine*
Post translational modification by Fic Protein targeting RAB GTPase (e.g. AnkX)
RAB<>GDP
GEF
GDI
GDI
C
EF-Tu<>GTP
EF-Tu<>GDP
EF Tu<>GDP
Fic
tRNA
Phosphate*
Post translational modification by Fic Protein targeting EF-Tu GTPase (e.g. Doc)
Translation of mRNA
tRNA
Block of Translation of mRNA = cell death

Figure 1-2 depicts the mechanism by which Fic proteins induce their toxic effect on different GTPase controlled cellular pathways. In 2-A Fic proteins that target Rho GTPases are shown; Rho GTPases are regulators of actin cytoskeleton, Fic proteins affect Rho GTPases by post-translational modification (in this example VopS adds an AMP to GTP-loaded Rho GTPase to hydroxyl containing group at the target site). This modification prevents the Rho GTPase from exerting its function on its downstream effectors with consequential collapse of the actin cytoskeleton. In 2-B, the action of Fic proteins targeting Rab GTPases is shown (AnkX as an example). Rab GTPases are activated by Guanine Exchange factors, and are dissociated from the cell membrane by Guanine Dissociation Inhibitors. Fic proteins introduced into the cell block both interactions by catalyzing a reaction to add a phosphate-containing molecule (Phosphocholine in the case of AnkX) to hydroxyl containing amino acid target of the Rab GTPase. This PTM on Rab results in vesicular transport interruption and promotion of establishing bacteria-containing vacuoles inside the cell. In 2-C, the effect of the Fic domain containing bacterial toxin Doc is shown. It exerts its catalytic function by addition of phosphate to its target EF-Tu (elongation factor Tu) which inhibits the formation of the active form of EF-Tu which in turn prevents protein translation and resulting in eventual cellular death. This figure was adapted from [41]

1.4.3.5 Eukaryotic Fic Domain Bearing Proteins

HYPE (**H**untingtin **y**east interacting **p**rotein **E**) protein in eukaryotes is a highly conserved protein bearing a Fic domain. It has been identified even before bacterial Fic proteins were identified; however, recently the function of HYPE has been elucidated further, and the relationship of its Fic domain to cell signalling has been shown. HYPE was shown to be essential for visual transmission in neuroglial cells, carrying out this function by means of post translational modifications (by addition of AMP) on its target protein (heat shock protein 70 type chaperone BiP of the endoplasmic reticulum, a core regulator of the cellular response against misfolded proteins)[66-68] The crystal structure study also suggests that this protein does not have a predilection to small GTPases, unlike most of bacterial Fic proteins [69].

This mechanism was also shown to be utilised by a roundworm orthologue of HYPE (Termed as FIC-1 of *Caenorhabditis elegans).* The in vivo study utilizing a mutant version of FIC-1 versus the wild type form in *C. elegans,* showed that FIC-1 mutation does not affect the lifespan of the worm; however, it makes it significantly more susceptible to be killed by *Pseudomonas aeruginosa* [70].

1.4.3.6 The connection with PAK proteins

PAK proteins (p21 activated kinases) are effectors activated by Cdc42 and Rac Rho GTPases. Their main role is on the regulation of actin polymerisation of the cytoskeleton, which in turn influence cellular motility, adhesion, migration, and phagocytosis and shape or morphology [57-59, 71, 72]. The group of PAK proteins related to Cdc42 and Rac (the main Rho GTPases affected by Fic domain bearing proteins) are PAK1 and PAK2 [71, 72].

The role of PAK proteins and especially PAK1 and PAK2 in actin cytoskeleton regulation are complex; however, their functions can be summarised in three points:

First, PAK1 and PAK2 affect the dissolution of stress fibre formation, which in turn is related to cellular adhesion and motility [59]) as well as being detrimental to the formation of focal adhesions a role that is mainly controlled by Cdc42) [72, 73].

Second, and perhaps the more detrimental function, is the formation of filopodia. Filopodia are the major structures formed by the cell that allows cellular migration and motility, including chemotaxis. If disrupted, it would have a disruptive effect on clearing infections by hindering the migration of immune cells reaching the target area [74]. The function of these two PAKs is sometimes complementary, while they seem to have antagonizing functions in other instances suggesting that PAK2 may perhaps have a regulatory role on PAK1. However, these functional roles have shown to be reversed in other tissue types illustrating the complexity of this system and the need for further

research to connect the function of these proteins with infection propagation and not only cancer formation [73, 75, 76].

A more specific role that is described for PAK2 revealing the differences between the two PAK proteins, it is the ability of PAK2 to induce cytostasis by halting the cellular transcription mechanism. This is achieved by means of Myc protein phosphorylation. It has been demonstrated that PAK2 (activated by Cdc42) directly phosphorylates Myc protein, which blocks Myc protein's ability to bind DNA and commence transcription and cellular proliferation [56, 77].

1.5 Bacterial Secretion Systems and Fic Proteins

1.5.1 Overview of Secretion Systems and Fic proteins

Secretion systems in bacteria have been shown to function in translocating proteins or DNA from inside the bacteria to either the outer membrane of the bacteria or the extracellular space. Some secretion systems can also "inject" their secreted proteins into other bacterial cells or even eukaryotic cells.

The reason for the translocation of secreted substrates can vary. DNA translocation by secretion systems between bacteria can help disseminate genetic material across bacterial species to promote survival (promoting resistance to certain chemical structures such as antibiotics, or genes to improve fitness in a certain environment) [78, 79]. Protein translocation between bacteria can help one strain of bacteria to out-compete another or interspecies competition by translocating toxic substances that have deleterious effects on the receiving bacteria [80]. Extracellular secretion of substances can help some bacteria form biofilms, which helps them maintain survivability in hostile environments and improve their pathogenicity by shielding them from hosts' defences [81-83]. Secretion of protein into eukaryotic cells (such as Fic protein) help the invader to promote or maintain its infection or cause cellular death or significant harm to the host (such as exotoxins) [84-87].

T3SS and T4SS have been the common mechanisms by which bacteria secrete Fic proteins (Table 1.2), both these systems span the Inner and outer membrane of Gram-negative bacteria and reach with their machinery to the membrane of the host cell (figure 1.3).

1.5.2 Secretion Systems in *Klebsiella pneumoniae*

The genomes of many sequenced strains of *K. pneumoniae* harbour more than one type of secretion systems Type IV [88], Type II [89] Type VI [90] secretion systems have all been shown to be present in this bacteria. Not all sequenced strains harboured the T4SS (VirB) system; however, other types of T4SS which are not dependent on the VirB system might be present instead (usually function in DNA uptake or pathogenic protein transfer) [91]. Most if not all strains, harbor the T2SS and T6SS in their genomes [92] but none harbor T3SS [93].

Table 1-2 lists some of the described Fic proteins and secretion system which has been implicated in the secretion of these proteins. Each bacterium in which the protein was described, is indicated in parenthesis adjacently.

Type IV Secretion System	Type III Secretion System	Other mechanisms
VbhT (*Bartonella schoenbuchensis*)[52, 94]	AvrAC (*Xanthomonas campestris*)[54, 95]	IbpA (*Histophilus somni*) Two way secretion system (T5SS)[53]
VopS (*Vibrio parahaemolyticus*)[43]	AvrB (*Pseudomonas syringae*)[39, 50, 62]	
BepA using VirB system (*Bartonella henselae*)[49]		
AnkX (*Legionella*)[40, 42]		
CBU2078 (*Coxiella burnetii*)[96]		
DrrA (*Legionella pneumophila*)[55] T4SS (?)		

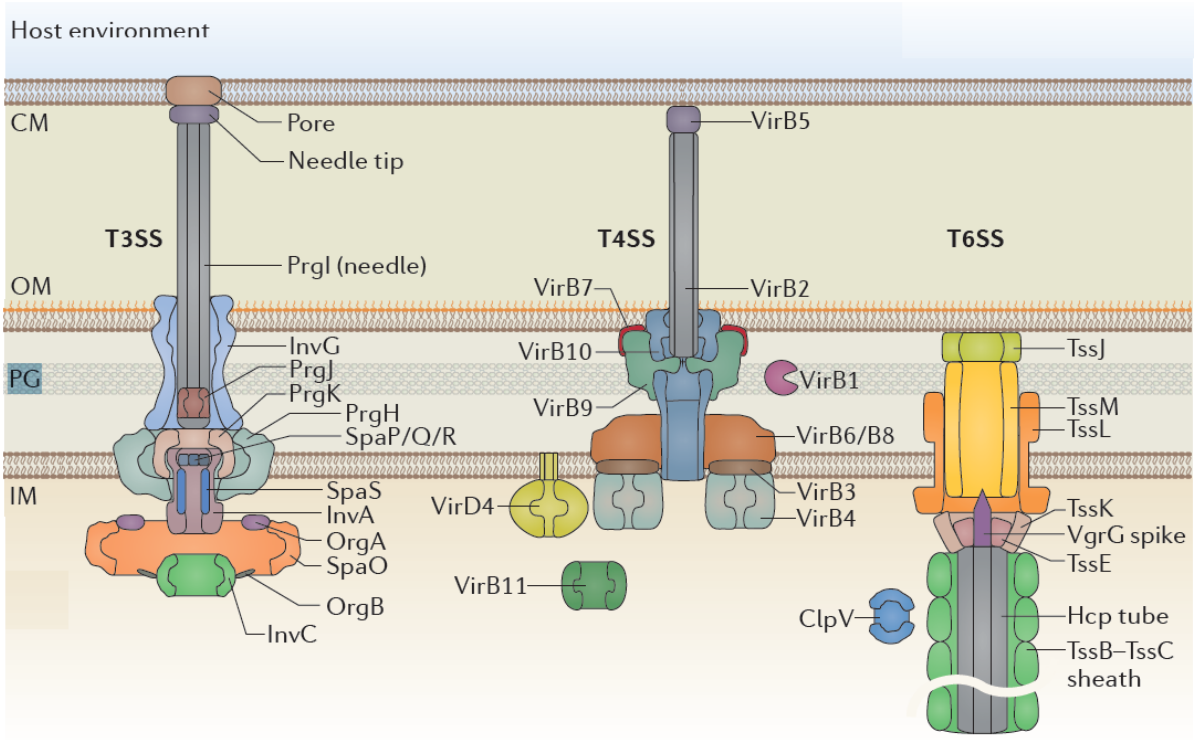


Figure 1-3 shows the structure of the Type III , Type IV and Type VI Secretion Systems which are all present in *K. pneumoniae*, CM indicates the cytoplasmic membrane of host cells, OM indicated the outer membrane of the bacteria, PG is the peptidoglycan layer and IM is the inner membrane of the bacteria. In T3SS PrgI forms the needle which pokes into the host cell, while InvC is the ATPase that powers the secretion mechanism, SpaS is the inner switch that is used to allow secretion, many other proteins identified act as structural proteins for the mechanism as well as chaperone proteins that identify proteins for secretion. In T4SS the VirB4 and VirB11 and VirD4 provide energy to the apparatus, VirB6-10 are core structural complex proteins while the rest form the pilus for the apparatus that form the length of the secretion system into the host cell. T6SS is usually described as an inverted phage, owing to the close relationship to the genes coding for this system and genes coded on bacterial phages. The figure was taken and modified from [97].

1.5.3 Type IV Secretion Systems

T4SS are one of the most diverse and well-studied secretion systems. They are unique in the way that they are used for protein and DNA transfer and are present in both Gram positive and Gram negative bacteria and some *archaea* as well [98]. The structure of T4SS traverses both the inner and outer membranes of the bacteria (where present) and deliver DNA or proteins into the target cell. They are used for conjugation (the delivery of DNA material between bacteria); this is a major mechanism of horizontal gene transfer and sex pili and the dissipation of genetic material within bacterial species that encode virulence factors and drug resistance. The dissemination of DNA molecules has been shown to be caused (in part) by conjugative plasmids that host genes encoding for T4SS and thus are able to induce their own delivery into other bacteria carrying with them other valuable genes that promote antibiotic resistance or spread of virulence factors [99]. This type (VirB type) of T4SS is but one type of the many types of this intricate secretion system, another type of T4SS that is instead been shown to aid in DNA transformation (been shown in *Helicobacter pylori* ComB based T4SS)[91].

Yet another type of T4SS is present in many pathogenic bacteria and is used by the bacteria to inject toxic proteins into the host cells that promote the infection of the invading bacteria or alter the cellular structure of the host to evade host's defences[100].

All three types of these systems are identified by the genes encoding for the ATPases that power the translocation of substances from the bacterial side, namely VirB4, VirB11 and VirD4 [91]in *Klebsiella*. There are many subclasses of T4SS that are distantly related but have many similarities between them. Most these subclasses are coded on single operons that identify them. Homology of the proteins for each part of the secretion apparatus (membrane pore, transmembrane cylinder or ATPase) can be identified between these different subclasses, however, the VirB4 ATPase seems to be almost ubiquitous amongst these different subclasses of T4SS[101].

1.5.4 Type VI secretion systems

T6SS have been described in many Gram-negative bacteria, including *K. pneumoniae*. T4SS and T6SS have the functional similarity in that they are used to inject toxic effectors into the host cells. While T4SS are usually permanent structures, T6SS are formed, then used to inject their effectors, and then rapidly recycled back in the cytosol of the predator bacteria. The structure and mechanism of action of these systems is homologous to phages, albeit in an upside-down fashion (where the bacteria are using this phage-like structure to inject its own functions in competition, rather than being on the receiving end of an invading phage) [102]. These systems are not only used to inject effectors into eukaryotic cells, but some bacteria also use these systems among themselves to attack other bacterial strains or species, and thus T6SS exhibit anti eukaryotic and anti-bacterial functions[103].

1.6 Aims and Objectives

The aims of this study are to identify Fic domain bearing proteins in *K. pneumoniae,* the study will then aim to characterize the identified proteins and attempt to verify if they adhere to the patterns seen in other pathogenic bacteria, namely to assess and characterize their role in the virulence of this bacteria. The rationale behind this endeavor is to characterize the function of the Fic protein to the virulence of *K. pneumoniae,* and to assess their individual roles that they play. This in turn will help formulate treatment options to tackle these dangerous bacteria.

Objectives:

To identify the genes coding for different homologs of Fic domain bearing proteins in *K. pneumoniae*,

To identify which of the identified Fic proteins is secreted,

To identify the secretion system used for secreting the Fic protein(s),

To assess the role of Fic proteins in virulence and possible mechanisms.

Hypothesis: given the literature of the many roles that Fic proteins play in the virulence of other bacteria; the hypothesis is that searching for Fic proteins in *K. pneumoniae* will show the presence of these proteins. Moreover, it is likely that these proteins play a significant role in the virulence of *K. pneumoniae.*

Chapter 2
Materials and Methods

2.1 Basic Microbiology Methods

2.1.1 Bacterial strains, Plasmids, Growth Conditions

All bacterial strains used were stored using 30% vol/volglycerol -brain heart infusion stock medium and kept in -80^{o} C freezers. Recovery of these strains was done first on Luria Agar (LA) plates (with antibiotics when appropriate) in a 37 °C incubator, with bacteria streaked to a single colony purity. Then a single colony was picked and grown in 5 ml Luria-Bertani (LB) broth in a 30 ml universal tube under aerobic conditions at 37 °C. The tube was placed in a shaking incubator at 200 RPM (unless otherwise specified), while also being supplemented with the appropriate concentration of antibiotics when applicable, as indicated in the description shown in table 2.1. Plasmids constructed or used in this study are described in table 2.2. All growth media used in this study were sterilised by autoclave and/or filter sterilisation prior to each use (using syringe filter sterilizer 45 μm- Merck Millipore)

2.1.2 Antibiotic Susceptibility Profiling

An antibiotic profile was obtained for strains for selection purposes, and to determine the use of antibiotic markers for mutant construction or plasmid transformation. Antibiotics were chosen according to the British Society for Antimicrobial Chemotherapy (BSAC) manuals, with disc diffusion assay used as a general test. A more specific antimicrobial susceptibility profile for chosen antibiotic markers was later obtained using Minimum Inhibitory Concentration methods according to BSAC recommendations.

Table 2-1 Bacterial strains used in the study

Bacterial Strain	Description	Resistance	Source
Klebsiella pneumoniae strains			
K. pneumoniae HS04160	Clinical strain isolated from urine contains KpGI-2 Genomic Island	Ceftazidine, Cefuroxime, Ciprofloxacin, Ampicillin, Tobramycin Kanamycin	Lab 212 Archive by w of Dr Hong-Yo O Fudan University[104]
K. pneumoniae HS04160 pKOBEG-Apra	*K. pneumoniae* HS04160 +pKOBEG-apramycin, plasmid containing the red recombinase genes (used for construction of the 4 mutants below)	Apramycin	This study
K. pneumoniae HS04160Δ*fic-rl*,	HS04160 strain with a knockout deletion of the gene encoding for Fic domain bearing protein FIC-RL		This study
K. pneumoniae HS11286Δ*fic-rl*,	HS11286 strain with a knockout deletion of the gene encoding for Fic domain bearing protein FIC-RL		This study
K. pneumoniae HS04160Δ*docKp*	HS04160 strain with a knockout deletion of the gene encoding for Fic domain bearing protein DOCKp		This study
K. pneumoniae HS04160Δ*fic-gn*	HS04160 strain with a knockout deletion of the gene encoding for Fic domain bearing protein Fic-GN (Orf 5 of KpGI-2)		This study
K. pneumoniae HS04160Δ*KpGI-2*	HS04160 strain with a knockout deletion of the entire KpGI-2 genomic Island		This study
K. pneumoniae MGH78578	Fully sequenced strain Isolated in Washington University from a sputum sample		Lab 212 Archive

K. pneumoniae HS11286	Fully sequenced strain with multi-drug resistance isolated from human sputum in Shanghai, China		Lab212 Archive[105]
K. pneumoniae HS11286 ΔKPC ΔMDR	A mutant constructed with in-frame deletion of *blaKPC* (carbapenem resistance) on plasmid pKPHS2 and a multidrug resistance island on plasmid pKPHS3 between-KPHS_p300510 and KPHS_p300880 gene (gene IDs).		Lab 212 Archive (I Dexi Bi)
K. pneumoniae HS11286-*fic-ad*	HS11286 ΔKPC ΔMDR was transformed with plasmid pWSK29-Apra-*fic-ad* for secretion study	Apramycin	This study
K. pneumoniae HS11286-Fic-RL	HS11286 ΔKPC ΔMDR was transformed with plasmid pWSK29-Apra-*fic-rl* for secretion study	Apramycin	This study
K. pneumoniae HS11286 –*fic-re*	HS11286 ΔKPC ΔMDR was transformed with plasmid pWSK29-Apra-*fic-re* for secretion study	Apramycin	This study
K. pneumoniae HS11286-*docKp*	HS11286 ΔKPCΔMDR was transformed with plasmid pWSK29-Apra-*docKp* for secretion study	Apramycin	This study
K. pneumoniae HS11286-*fic-gn*	HS11286 ΔKPC ΔMDR was transformed with plasmid pWSK29-Apra-*fic-gn* for secretion study	Apramycin	This study
K. pneumoniae HS11286-*egfp*	HS11286 ΔKPCΔMDR was transformed with plasmid pWSK29-Apra-*egfp*	Apramycin	This study
K. pneumoniae HS04160- *fic-rl*	HS04160 was transformed with plasmid pWSK29-Apra-*fic-rl* for secretion study	Apramycin	This study
K. pneumoniae HS11286 ΔMDR ΔKPC ΔCPS	*K. pneumoniae* HS11286 ΔKPCΔMDR with an additional inframe deletion of the genes responsible for the production of the capsular polysaccharides (Genes in cluster between gene *galF-ugd)* for easier manipulation		Lab 212 (Yingzhou Xie, visiti from Fudan University
K. pneumoniae HS11286 ΔCPS-*fic-rl*	HS11286 ΔMDR ΔKPC ΔCPS was transformed with plasmid pWSK29-Apra-*fic-rl* for secretion study	Apramycin	This study

K. pneumoniae HS11286 ΔKPCΔMDR ΔT6SS1 ΔT6SS3	A mutant constructed with an inframe deletion of both clusters (1 and 3) that contain type 6 secretion system components.		Lab 212 (Dav Ngmenterebo)
K. pneumoniae HS11286 ΔT6SS1 ΔT6SS3- *fic-rl*	HS11286 ΔKPCΔMDR ΔT6SS1 ΔT6SS3A mutant was transformed with plasmid pWSK29-Apra-*fic-rl* for secretion study	Apramycin	This study
K. pneumoniae HS11286 *ΔMDRΔKPCΔCPSΔICE1*	HS11286 ΔMDR ΔKPC ΔCPS strain used to construct an inframe deletion of ICE1 genes ID designation (ICEKpnHS11286-1)[105]		Lab 212 (Robeena Farzand)
K. pneumoniae HS11286 *ΔICE1-fic-rl*	HS11286 ΔMDRΔKPCΔCPSΔICE1 was transformed with plasmid pWSK29-Apra-*fic-rl* for secretion study	Apramycin	This study
K. pneumoniae HS11286 *ΔMDR ΔKPC ΔCPS Δmob1*	HS11286 ΔMDR ΔKPC ΔCPS strain used to construct an inframe deletion of *mob1* gene only in the element ICE1		Lab 212 (Robeena Farzand)
K. pneumoniae HS11286 *Δmob1-fic-rl*	HS11286 ΔMDR ΔKPC ΔCPS ΔMob1 was transformed with plasmid pWSK29-Apra-*fic-rl* for secretion study	Apramycin	This study
K. pneumoniae HS11286 *ΔMDRΔKPCΔcpsΔICE2*	HS11286 *ΔMDRΔKPCΔcpsΔICE2* strain used to construct an inframe deletion of ICE2 element (ICE 2 has the gene ID designation (ICEKpnHS11286-2)[105])		Lab 212 (Robeena Farzand)
K. pneumoniae HS11286 *ΔICE2 fic-rl*	HS11286 ΔMDRΔKPCΔCPSΔICE2 was transformed with plasmid pWSK29-Apra-*fic-rl* for	Apramycin	This study
K. pneumoniae HS11286 *ΔMDR ΔKPC ΔCPS Δmob2*	HS11286 ΔMDR ΔKPC ΔCPS strain used to construct an inframe deletion of *mob2* gene only in the element ICE2		Lab 212 (Robeena Farzand)
K. pneumoniae HS11286 *Δmob2Fic-RL*	HS11286 ΔMDR ΔKPC ΔCPS ΔMob2 was transformed with plasmid pWSK29-Apra- *fic-rl* for secretion study	Apramycin	This study
K. pneumoniae HS11286 ΔMDRΔKPCΔcpsΔvirB(1-2)ΔvirB(4-6)::hph	HS11286 ΔMDR ΔKPC ΔCPS strain used to construct an inframe deletion of genes responsible for the cylindrical part of the Type 4 Secretion system on ICE1 conjugative element virB(1-2)ΔvirB(4-6), this is a marked mutant	Hygromycin	Lab 212 (Yingzhou Xie, visiti from Fudan

K. pneumoniae HS11286 ΔCPSΔvirB(1-2)ΔvirB(4-6)- *fic-rl*	*K. pneumoniae* HS11286 ΔMDRΔKPCΔcpsΔvirB(1-2)ΔvirB(4-6)::hph strain was transformed with plasmid pWSK29-Apra- *fic-rl* for secretion study	Apramycin	This study
Escherichia coli strains			
Escherichia coli S17-λ*pir*	Strain lysogenized with the *λpir* phage to host plasmid that have a R6K origin of replication, used for introduction of suicide vectors by means of conjugation using RP4 based conjugation		Lab 212 Archive[106]
E. coli CC118λ*pir*	Strain lysogenized with the *λpir* phage to host plasmid that have a R6K origin of replication, used for cloning procedure		Lab 212 Archive[106]
E. coli DH5α	Bacterial host used for cloning procedure		Promega
E. coli BL21(DE3)	Bacterial host used for protein expression for plasmids under T7 promoter, also is lysogenized with the Lambda-DE3 phage that contains the T7 polymerase, this strain represses protein production in the absence on the induction agent (IPTG)		Lab107 Archive

Table 2-2 Plasmids constructed or used in this study.

Plasmid	Description	Source
pJTOOL-3	Used to construct suicide vectors, pDS132 derivative with R6K pir protein dependant promoter giving the ability to select cells that do not lose the plasmid, *sacB* sucrose counterselection marker, *cat* : chloramphenicol resistance gene.	Lab 212 Archive [106]
pJTOOL-8	Similar to pJTOOL-3 with an apramycin resistance cassette flanked by Flippase Recognition Sites (FRT) for later excision using Flippase containing plasmid (pFLP2-Apra)	Lab 212 Archive [106]
pMA-KpGI-2-KO-Apra	The constructed suicide vector to carry out KO of KpGI-2 from pJTOOL-3, with apramycin resistance cassette	This study
pKOBEG-Apra/pKOBEG-Hygro	Lambda red genes with an arabinose promoter site, apramycin cassette added for positive selection, selection with Apramycin at 30ug/ml.	Lab 212 Archive
pWSK29-Apra	A plasmid previously used for protein expression in *K. pneumoniae,* Apramycin gene cassette added. selection with Apramycin at 30ug/ml.	Lab 212 Archive
pWSK29-Apra-*fic-ad*	pWSK29-Apra with the *fic-ad* gene cloned inframe from start to stop codon in the multiple cloning site using HindIII restriction site on forward primers and EcoRI on reverse primers, digested and ligated into these sites on the plasmid FLAG TAG added on reverse primers (C Terminus).	This study
pWSK29-Apra-*fic-rl*	pWSK29-Apra with the *fic-rl* gene cloned inframe from start to stop codon in the multiple cloning site using HindIII restriction site on forward primers and EcoRI on reverse primers, digested and ligated into these sites FLAG TAG added on reverse primers (C Terminus).	This study

pWSK29-Apra-*fic-re*	pWSK29-Apra with the f*ic-re* gene cloned inframe from start to stop codon in the multiple cloning site using HindIII restriction site on forward primers and EcoRI on reverse primers, digested and ligated into these sites on the plasmid, FLAG TAG added on reverse primers (C Terminus).	This study
pWSK29-Apra-*docKp*	pWSK29-Apra with the *docKp* gene cloned inframe from start to stop codon in the multiple cloning site using HindIII restriction site on forward primers and EcoRI on reverse primers, digested and ligated into these sites on the plasmid FLAG TAG added on reverse primers (C Terminus).	This study
pWSK29-Apra-*fic-gn*	pWSK29-Apra with the *fic-gn* gene cloned in frame from start to stop codon in the multiple cloning site using HindIII restriction site on forward primers and EcoRI on reverse primers, digested and ligated into these sites on the plasmid FLAG TAG added on reverse primers (C Terminus).	This study
pWSK29-Apra-*egfp*	pWSK29-Apra with the *egfp* gene cloned in frame from start to stop codon in the multiple cloning site using HindIII restriction site on forward primers and EcoRI on reverse primers, digested and ligated into these sites on the plasmid No FLAG tag, used only for confirmation of protein expression by ultraviolet light observing eGFP.	This study
pRK5-myc	A commonly used high copy number eukaryotic expression plasmid with a strong CMV promoter and a myc tag on the C terminus following the multiple cloning sites, selection with 100ug/ml Ampicillin.	Lab 107 Archive. GE Healthcare Life sciences
pRK5-myc –*fic-ad*	pRK5-myc- with the *fic-ad* gene cloned in frame from start to stop codon in the multiple cloning site using BamHI restriction site on forward primers and EcoRI on reverse primers, digested and ligated into these sites on the plasmid.	This study

pRK5-myc –*fic-rl*	pRK5-myc- with the *fic-rl* gene cloned in frame from start to stop codon in the multiple cloning site using BamHI restriction site on forward primers and EcoRI on reverse primers, digested and ligated into these sites on the plasmid.	This study
pRK5-myc –*fic-re*	pRK5-myc- with the *fic-re* gene cloned in frame from start to stop codon in the multiple cloning site using BamHI restriction site on forward primers and EcoRI on reverse primers, digested and ligated into these sites on the plasmid.	This study
pRK5-myc –*docKp*	pRK5-myc- with the *DocKp* gene cloned in frame from start to stop codon in the multiple cloning site using BamHI restriction site on forward primers and EcoRI on reverse primers, digested and ligated into these sites on the plasmid.	This study
pRK5-myc –*fic-gn*	pRK5-myc- with the f*ic-gn* gene cloned in frame from start to stop codon in the multiple cloning site using BamHI restriction site on forward primers and EcoRI on reverse primers, digested and ligated into these sites on the plasmid.	This study
pRK5-myc –*fic-rl-HA*	pRK5-myc- with the *fic-rl* gene cloned in frame from start to stop codon in the multiple cloning site using BamHI restriction site on forward primers and EcoRI on reverse primers, digested and ligated into these sites on the plasmid. This is a mutant version cassette of the Fic-RL gene with the conserved histidine residue in the FIC motif replaced with an Alanine residue.	This study
pFLP2-Apra	Plasmid coding for the Flippase gene for converting K.O. mutants to markerless mutants by excising DNA flanked by the FRT site introduced on each flank (used to construct different versions).	Lab 212 Archive
pGEX-4T-1	A GST expression vector that adds a GST tag to the N terminus to the expressed protein with a proteolytic site for later cleavage of the GST using thrombin, a *tac* promoter, l*aqlq* repressor.	This study

pGEX-4T-1-*fic-rl*	pGEX vector carrying the sequence of the *fic-rl* gene introduced in the MCS that would add a GST tag to the N terminus of the protein produced	This study
pGEX-4T-1-*fic-rl*-HA	pGEX-4T-1 vector carrying a mutant version of *fic-rl* gene in the multiple cloning site, Mutant version cassette of the *fic-rl* gene was constructed using SOE-PCR to introduce two point mutations that would result in replacing the conserved Histidine residue with an Alanine	This study

Table 2-3 Primers designed or used in this study

Primer Name (orientation)	Description	features	Sequence	Replicon size (bp)	Referenc
KpGI-2 P1- F	Amplify Left flanking region of KpGI-2 KO SOE product	PstI capped restriction site(highlighted)	ATACTGCAGCGCGGCGATTACGGTTTAAT	444	This study
KpGI-2 P2- R		5' sequence identical to GmR (highlighted)	CTTCAAGATCCCCAATTCGGTCATTCAGAGTCACATGAG		This study
GmF	primers to amplify antibiotic cassettes from a common flanking region in multiple vectors	Cassette includes the FRT sites	TCAGAGCGCTTTTGAAGCTAATTCG	1400	This study
GmR			AGGAACTTCAAGATCCCCAATTCG		This study
KpGI-2 P5 -F	Amplify Right flanking region of KpGI-2 KO SOE product	5' sequence identical to GmF (highlighted)	GAGCGCTTTTGAAGCTAATTCGAGCCATGATGTTTAGTTATCTC	580	This study
KpGI-2 P6 -R		SalI capped restriction site(highlighted)	GCGGTCGACTGGCGAGAAAATGGCGAT		This study
KpGI-2 screen-F	Primers outside the flanking region to screen for mutation		CATCTGATAGTGCGTCTGCTG	Wt=6.3k Mut.=2.5k	This study
KpGI-2 screen-R			GCTATTTGGCCATCTGGAGGG		This study
FIC-AD-F	Detect the AD Fic homolog(universal)		GGCGTCACTCGCGATTG	328	This study
FIC-AD-R			CAGTTGGCTGGAACAC		This study
FIC-DOC-F	Detect the Doc Fic homolog(universal)		CTGTTTCACGACAGGCTGC	281	This study
FIC-DOC-R			GCCTCGACGGTCATGCC		This study
FIC-RE-F	Detect the *fic-re* homolog(universal)		GCTGGGGATTGCCGAT	436	This study
FIC-RE-R			TTGATAATCCAGCGGCC		This study
FIC-RL-F	Detect the *fic-rl* homolog(universal)		ATTCATCACCAGTTGTATC	345	This study
FIC-RL-R			TCTGCTGGCAGGCCGCACT		This study
KpGI-2 Orf5-F	Detect the Kpgi-2 Orf5 Fic homolog		ATGTTCACCCTATCCTGTTT	640	This study
KpGI-2 Orf5-R			CCCTGCTCTGTTCCCTTAT		This study

Fic RL KO P1	Amplify Left flanking region of *fic-rl* homolog- SOE product		GTAGCCTCGACAAGTTTACGTC	1001	This study
Fic RL KO P2		5' sequence identical to GmR2	AGGTACTTCAAGATCCCCAATTTAGAATAGCCCGGTTTTGATTG		This study
Fic RL KO P5	Amplify Right flanking region of *fic-rl* homolog- SOE product	5' sequence identical to GmF	TCAGAGCGCTTTTGAAGCTAATTCGCATACTCCCTCCGCAACGTCG	1004	This study
Fic RL KO P6			CACGCTTGTTCGCTGCAAAG		This study
Fic RL Screen-F	Primers outside the flanking region to screen for mutation		ATAAACACCACGCGCTCG	WT=2.6kb Mu=3.7kb	This study
Fic RL Screen-R			GAAATGGGTTATCTTTTGCATG		This study
Fic Doc KO P1	Amplify Left flanking region of *fic-rl* homolog- SOE product		CGGCGCTCAGGCTCTGCTCGTAG	1001	This study
Fic Doc KO P2		5' sequence identical to GmR2	AGGTACTTCAAGATCCCCAATTTGACGGTTAACTCGTTGCAAC		This study
Fic Doc KO P5	Amplify Right flanking region of *fic-rl* homolog- SOE product	5' sequence identical to GmF	TCAGAGCGCTTTTGAAGCTAATTCGCATTTATCCGCAAGCTCCCTG	1004	This study
Fic Doc KO P6			GCGGCTATGTGCTGGAGAACGGCC		This study
Fic Doc Screen-F	Primers outside the flanking region to screen for mutation		GGTACGAACTGCGATTCATC	WT=2.6k Mu=3.7k	This study
Fic Doc Screen-R			CGCCGATTATCATTCAGCAG		This study
SacB – F	Detect *sacB* genes present on pJTOOL-3		TAACAGCAGCGTGACAAGTGTAGGCCCGTAGTCTGCAAAT	1636	This study
SacB – R			GCCCTATGGGATTCACCTTT		This study
EBGNHe	Detect Lambda red genes on pKOBEG		CCCGCTAGCGAAAAGATGTTTCGTGAAGC	1960	This study
EBGh3			GGGAAGCTTATTATCGTGAGGATGCGTCA		This study
pWSK29-Apra-AD-F	Primers to amplify *Fic-ad* gene and add restriction sites for cloning purposes into pWSK29	(HindIII restriction site, one bp added to 5'end in order to be in frame with MCS in *lacZ*	TTAAAGCTTC.ATGAGCCGTTACCAACCGCC	1002	This study
pWSK29-Apra-AD-R		EcoRI restriction sitem FLAG TAG nucleotide sequence	TTAGAATTCCTACTTCTTGTCGTCATCGTCTTTGTAGTCAACCAGCCAGCTTCCACC		This study

pWSK29-Apra-RL-F	Primers to amplify *Fic-rl* gene and add restriction sites for cloning purposes into pWSK29	(HindIII restriction site, one bp added to 5'end in order to be inframe	TTAAAGCTTCATGAGCGATAAATTCGGCGATG	603	This study
pWSK29-Apra-RL-R		EcoRI restriction site FLAG TAG nucleotide sequence	TTAGAATTCCTACTCCTTGTCGTCATCGTCTTTGTAGTCAGATTCCCGCGCTTCGCTTAC		This study
pWSK29-Apra-RE-F	Primers to amplify *Fic-re* gene and add restriction sites for cloning purposes into pWSK29	(HindIII restriction site, one bp added to 5'end in order to be in frame	TTAAAGCTTC.ATGGCTAATAAACTCGGGATCGC	555	This study
pWSK29-Apra-RE-R		EcoRI restriction sitem FLAG TAG nucleotide sequence	TTAGAATTCCTAATCCTTGTCGTCATCGTCTTTGTAGTCTCTTCTTAAGGCCTTACTG		This study
pWSK29-Apra-DOCKp-F	Primers to amplify *docKp* gene and add restriction sites for cloning purposes into pWSK29	(HindIII restriction site, one bp added to 5'end in order to be in frame	TTAAAGCTTCATGACGCTGCAGATTATCTCAG	369	This study
pWSK29-Apra-DOCKp-R		EcoRI restriction sitem FLAG TAG nucleotide sequence	TTAGAATTCTCATCCCTTGTCGTCATCGTCTTTGTAGTCACGCAAACGCGCGACAATCTG		This study
pWSK29-Apra-GN-F	Primers to amplify *Fic-gn* gene and add restriction sites for cloning purposes into pWSK29	(HindIII restriction site, one bp added to 5'end in order to be in frame	TTAAAGCTTCATGGCTCAATTTAATCACTTTG	1143	This study
pWSK29-Apra-GN-R		EcorI restriction sitem FLAG TAG nucleotide sequence	TTAGAATTCTCACAACTTGTCGTCATCGTCTTTGTAGTCGTCAGGAATAAACCCCTCGTC		This study
pWSK29-Apra-eGFP-F	Primers to amplify *egfp* gene and add restriction sites for cloning purposes into pWSK29	(XbaI restriction site, one bp added to 5'end in order to be in frame	TTAAAGCTTCATGCGTAAAGGAGAAGAAC	717	This study
pWSK29-Apra-eGFP-R		EcoRI restriction site	TTAGAATTCTTATTTGTATAGTTCATCCATGC		This study
M13 Primer F	Primers outside the multiple cloning site of pWSK29 plasmid		CGCCAGGGTTTTCCCAGTCACGAC	217bp for empty vector	Common sequencir primers MCS of containin vectors
M13 Primer R			AGCGGATAACAATTTCACACAGGA		

FicRL(H-A)F	Used alongside Reverse primers amplifying (pGEX4T-1,pRK5-myc) the *Fic-rl* gene to create a half-length segment with mutation introduction	a point mutation introduced in the primer changing the sequence of conserved Hisidine to Alanine, used for SOE PCR	GAAATTAATGTGCTGGCCCCTTTTCGTCTTGG	419	This study
FicRL(H-A)R	Used alongside pGEX-4T-1-FicRL-F or amplifying the *Fic-rl* gene to create a half-length segment of the gene while introducing a point mutation	a point mutation introduced in the primer sequence changing the sequence of conserved Hisidine to Alanine of the *Fic-rl* gene, used for SOE PCR	CCAAGACGAAAAGGGGCCAGCACATTAATTTC	216	This study
pGEX-4T-1 MCS-F	Screening primer of the multiple cloning site		GGCAAGCCACGTTTGGTG	202 for empty vector	This study
pGEX-4T-1 MCS-R			GTAAGCGGATGCCGGGAG		This study
pGEX-4T-1-FicRL-F	Used to amplify *Fic-rl* gene for purpose of cloning the sequence into pGEX-4T-1 vector	BamHI restriction site	ATGAGCGATAAATTCGGCGATG		This study
pGEX-4T-1-FicRL-R		EcoRI restriction site	CTACTCAGATTCCCGCGCTTCG		This study
pRK5-myc-FicAD-F	Primers to amplify *Fic-ad* gene and add restriction sites for cloning purposes into pRK5-myc		CTCGGATCCATGAGCCGTTACCAACCGCC	1002	This study
pRK5-myc-FicAD-R			CTCGAATTCCTACTTAACCAGCCAGCTTCCAC		This study
pRK5-myc-FicRL-F	Primers to amplify *fic-rl* gene and add restriction sites for cloning purposes into pRK5-myc		CTCGGATCCATGAGCGATAAATTCGGCGATG	603	This study
pRK5-myc-FicRL-R			CTCGAATTCCTACTCAGATTCCCGCGCTTCG		This study
pRK5-myc-FicRE-F	Primers to amplify *fic-re* gene and add restriction sites for cloning purposes into pRK5-myc		CTCGGATCCATGGCTAATAAACTCGGGATCGC	555	This study
pRK5-myc-FicRE-R			CTCGAATTCCTAATCTCTTCTTAAGGCCTTACTG		This study

pRK5-myc-DOCKp-F	Primers to amplify *docKp* gene and add restriction sites for cloning purposes into pRK5-myc		CTCGGATCCATGACGCTGCAGATTATCTCAGCGG	369	This study
pRK5-myc-DOCKp-R			CTCGAATTCTCATCCACGCAAACGCGCGAC		This study
pRK5-myc-FicGN-F	Primers to amplify *fic-gn* gene and add restriction sites for cloning purposes into pRK5-myc		CTCGGATCCATGGCTCAATTTAATCACTTTGAAC	1143	This study
pRK5-myc-FicGN-R			CTCGAATTCTCACAAGTCAGGAATAAACCCCTC		This study

2.2 DNA Manipulation Methods:

2.2.1 DNA Collection

Genomic DNA extraction, Plasmid preparation, and Gel extraction methods:

Genomic DNA extraction was achieved using the ArchivePure DNA Cell/Tissue and Tissue Kits (5 Prime) according to the manufacturer's instructions. Following overnight incubation of the 1 ml bacterial culture, and the bacterial cells were lysed using an anionic detergent buffer. Proteinase K was added afterwards, and then the sample was incubated with an addition of RNAse at 37 ^{o} C for 30 minutes. Samples were then centrifuged at 15000 g for 10 minutes, and the supernatant collected. The DNA was precipitated from the supernatant, by the addition 100% Isopropanol and was then pelleted by centrifugation. The pellet was then washed with 70% Ethanol and dried on the bench for 1 hour. Lastly, the DNA pellet was dissolved in ultra-pure water (DNAse free water).

Plasmid preparation was done using Sigma Aldrich GenElute plasmid preparation kit as per manufacturer's instructions. Following incubation, 3-5 ml of overnight bacterial culture was used; the bacteria were lysed. The resulting plasmid DNA was collected using provided columns which trap plasmid DNA from the samples, which was collected by means of elution using ultra-pure water.

Similarly, DNA samples extracted from agarose gel (PCR products, plasmid digestion reactions) were purified out of the agarose gel using agarose gel extraction kits (GenElute gel extraction kit-Sigma Aldrich) according to manufacturer's instruction.

2.2.2 DNA Quantification

DNA was quantified using the Nanodrop 2000 (Thermo Fisher scientific) spectrophotometer using elution buffer or ultra-pure water as blank (where appropriate).

2.2.3 DNA Amplification- Polymerase Chain Reaction (PCR) Methods

2.2.3.1 General PCR method

High fidelity polymerase enzyme (KOD Hotstart DNA polymerase-Merck Millipore) was used for cloning and mutagenesis work, whereas a lower fidelity enzyme (GoTaq G2 Green polymerase-Promega) was used for gene screening purposes (deoxynucleotide obtained from Bioline). Thermocyling temperatures were used according to manufacturer's instruction: 95 ^{o} C initial denaturation for 5 minutes, cycles are from 25-40 cycles, with each cycles having 95 ^{o} C denaturation for 10 seconds, primer annealing temperature for 10 seconds, followed by extension time at 72 ^{o} C for 1 minute /Kb, the following the cycles, the reaction was concluded with 10-minute extension at 72 ^{o} C)
Each PCR reaction was prepared in a master mix fashion which would contain all the elements of the reaction according to the protocol prior to adding template and primers. The specific concentrations of each component in each reaction would contain in a 50 µl reaction as follows: Polymerase 1 µl, 1.5 µl of antisense and sense primers (or 10 µM concentrations), dNTPS (2mM for each dNTP), 5 µl of 10x buffer, 3 µl of a stock solution of $MgSO_4$ (to 1.5mM final concentration) template DNA 50-100 *ng* and top the reaction mixture to 50 µl with PCR grade water).

2.2.3.2 Primer design

Table 2.3 shows the primers that were designed and used in this study. Primer design was done using (ApE) Plasmid Editor Software: (http://biologylabs.utah.edu/jorgensen/wayned/ape/). Each sequence was then verified for quality using Primer3 software (http://primer3.ut.ee/) which verifies certain quality parameters such as melting temperature, presence of secondary structures, primer dimers or hairpin loops.

2.2.3.3 Colony PCR

Colony PCR was used to assess the genotype of bacteria using a fresh colony. The colony to be tested was picked from the plates, and resuspended in 50 µl ultra-pure water and heated at 100^{o} C for 15 minutes to lyse the bacterial cells. The samples were then centrifuged at 12000g for 1 minute, and 4 µl of the supernatant was used as a template.

2.2.3.4 Splicing Overlap Extension (SOE) PCR

This PCR method is used to join 2 or more fragments of DNA with complementary overlapping sequences (introduced by primer design) into one fragment. The spliced DNA fragments with complementary sequences were joined together in a PCR reaction by allowing the PCR reaction to proceed without addition of primers for 10-15 cycles. This method creates longer templates which are made up of the aforementioned fragments with the complementary homologous sequences (when these segments denature and anneal they naturally anneal together). These long templates are then amplified by adding primers annealing at each end amplifying the entire fragment. 30-40 cycles were carried out at this point to increase the quantity of the now fused desired fragment. The result was confirmed using gel electrophoresis (methods were adapted from Heckman[107] and Choi[108]).

2.2.4 DNA Analysis by Agarose Gel Electrophoresis

DNA visualisation was achieved using agarose gel electrophoresis. Agarose gel was prepared by dissolving 0.8 g of agarose powder (Bioline) /100ml of TAE buffer (Tris-Acetic acid-EDTA) and later supplemented with Ethidium Bromide (5 µl of 10 mg/ml stock used for each 100 ml of gel). 6X DNA loading Dye was added (Thermo Fisher scientific) to the samples to a 1X concentration. Samples were then run alongside a DNA ladder (Generuler 1Kb ladder Thermo Fisher Scientific) for size estimation; the bands were visualised using a UV light chamber.

2.2.5 Mutant Construction

2.2.5.1 Electrocompetent cells preparation

Any bacterial strains to be transformed with plasmid or single stranded DNA was first prepared for electroporation (Strains selected for mutagenesis are detailed with rationale for selection in section 4.3.1.). Two specialized media were used in this process: Super Optimal Broth (SOB Media: 2% Tryptone w/v 0.5% w/v yeast extract,10 mM NaCl, 2.5 mM Potassium chloride ,10 mM $MgCl^2$,10 mM $MgSO^4$) or SOB and Super Optimal broth with Catabolite repression (SOC Media: SOB+ 20mM Glucose). SOC provides all necessary components for the growth of the bacteria that undergo the stressful electroporation conditions. Catabolite repression was shown to cause faster growth for bacteria when Glucose is present in the media, thus allowing the cells to recover quicker and improve transformation efficiency. All electrocompetent cell preparation was done in a 4 ^{o} C cold room or on ice Electrocompetent cells preparation protocol was adapted from Dower et al with recent additions from Gonzales et al [109, 110].

First, an overnight culture was prepared in a small flask which was then diluted at 1:100 and allowed to grow to an optical density at OD600nm of 0.5-0.6 OD units in 150 ml volume. Any induction with IPTG or L-Arabinose was then carried out with the dilution step at this point.

Bacteria were then pelleted by centrifugation at 4000g and resuspended in 50 ml sterilised and chilled 10% v/v glycerol, to remove excess ions in the solution. This wash step was repeated three times. Following the wash, the pellet was resuspended in 5ml of 10% glycerol, aliquoted into Eppendorf tubes at 80 µl volumes and stored in a -80°C freezer.

2.2.5.2 Transformation using electroporation

A vial of competent cells was retrieved from -80°C storage and the cells were allowed to thaw on ice. The desired DNA was then added (usually 50-100 ng for plasmid DNA,

200-500ng of ssDNA) and mixed thoroughly with the competent cells. The sample was placed in electroporation cuvette (Geneflow) and electroporated using 2.5kV setting using a Biorad *E.coli* Pulser. The bacteria were then immediately recovered with the addition of 900 µl of pre-warmed SOC broth and then allowed to incubate for in a shaking incubator for 1 hour at 37°C before being diluted and plated on appropriate plates.

2.2.5.3 Recombination methods

1- Lambda red recombination [111-113]:

(for diagram please refer to figure 4-3)

The lambda red recombination method was the primary mutant construction method employed in this study. This method utilises the red operon present on the phage λ *Red*; the genes of this operon (*exo, bet* and *gam*) are placed on temperature sensitive plasmid pKOBEG-Apra under the control of the arabinose induced promoter $P_{BAD.}$ Growing bacterial cells carrying this plasmid above 30°C causes the plasmid to be cured. The tightly regulated promoter $P_{BAD,}$ was induced by the addition of the monosaccharide L-Arabinose which allows the expression of the proteins encoded on the red operon. The function of these proteins in recombination is as follows: The Exo protein is an exonuclease that degrades the target site dsDNA in a 5'-3' direction while preserving the opposite direction thus preparing the area for Bet protein to bind in the presence of homologous single-stranded ssDNA (designed and constructed using SOE PCR and then introduced by means of electroporation). Bet protein directs a homologous recombination at the target site, while the Gam protein inhibits the host's nucleases (RecBCD exonucleases) to preserve the introduced ssDNA.

SOE PCR was used to manufacture the ssDNA used for the mutagenesis. The mutagenesis was designed to cause an inframe deletion of the target gene (from start to stop codon). A 400-600 bp regions upstream and downstream of the gene in question were amplified and then spliced with a gene for the antibiotic marker (e.g. Hygromycin). The recombination event planned using the red proteins removes the target gene in question from start to stop codon and replaces it with the antibiotic marker using the complementary homologous flanking regions. Bacterial cells that underwent this recombination event were selected with hygromycin and confirmed by PCR (by

preparing genomic DNA and testing it with primers that lay outside those 400-600 pb flanking regions).

Strains containing the (pKOBEG-Apra) were induced during the competent cells preparations steps and grown at 30°C with 30µl/ml Apramycin (confirmation by PCR was done at this point to verify the presence of the red operon genes).

Once these cells have been induced (and now contain the lambda red proteins within them), electrotransformation with the SOE product ssDNA would be successful; cells were allowed to recover for 1 hour at 37°C with SOC media and then spread on LA plates supplemented with Hygromycin - selection for the antibiotic marker introduced in the SOE PCR- at 37°C, which helps to cure the pKOBEG-Apra plasmid (later verified using colony PCR). The mutation was then confirmed by using PCR on genomic DNA using primers that lay outside the flanking regions on the genome sequence.

2- Allelic exchange [106] :

(for complete diagram please refer to figure 4-1 and 4-2)

For KpGI-2 deletion (*Klebsiella pneumoniae* genomic island 2), a knock out by deletion mutant was constructed using a conjugation based method that introduces a suicide vector containing a DNA segment, constructed by using overlap extension PCR (this method was adapted from Van Aartsen's method [106]). A suicide vector was constructed that carries DNA sequences prepared by SOE PCR made up of roughly 800bp homologous regions that flank the KpGI-2 Island sequence (upstream and downstream) with an antibiotic cassette gene encoding for (Apramycin) between these two flanks. This was aiming for a recombination effect that replaces the sequence of KpGI-2 that lays between these two flanks with the antibiotic cassette, this vector (pMA-KpGI-2-KO-Apra) was transformed into the transformable *E. coli* (CC118-λ pir), then confirmed by PCR (this is termed as a suicide vector as it cannot replicate inside a host that does not code for the λ pir protein present).

Plasmid pMA-KpGI-2-KO-Apra was then electroporated into the highly conjugable *E. coli* strain (S17-Iλ pir) for later conjugation. Colonies with the confirmed phenotype and genotype were selected.

S17-Iλ pir cells (as donor cells) that were confirmed to harbour the desired plasmid were recovered and streaked to purity, then grown in broth to an absorbance of OD600 of 0.6-0.8 (with appropriate antibiotics).The wild-type recipient strain was grown similarly.

The conjugation step included transferring the suicide plasmid to recipient *K. pneumoniae* strain HS04160. The donor and recipient were pelleted and washed twice with 10mM magnesium sulphate to remove any effectors in the media that might hinder the conjugation step (such as antibiotics). The two strains were then mixed and spotted on the nitrocellulose membrane (0.45 µm size pores) in a 1:1 ratio on LA plates and incubated overnight at 37°C.

After conjugation the cells were recovered from the membrane in 10 mM $MgSO_4$ solution (4 dilutions were prepared in 1/10 increments) they were plated on M9 minimal media [114]supplemented with appropriate antibiotics as follows:

<Donor-Donor> (no growth expected-confirmed)

<Recipient –recipient> (no growth expected-confirmed)

<Donor-Recipient> (colonies were detected)

M9 minimal medium plates were used as this medium suppresses the growth of modified *E.coli* strains and helps to select for *K. pneumoniae.* The addition of the antibiotics for the selection marker on the SOE product selects specifically for *K. pneumoniae,* which is what carries the plasmid of interest.

The colonies were then patched on M9 plates supplemented with 30 µl/ml Apra and M9 with 30 µl/ml Chloramphenicol (selects for the backbone of plasmid) plates. The patching was done to reduce the risk of *E. coli* carriage. The correct phenotype was then confirmed by PCR.

Allelic exchange can occur at any of the two homologous flanks, which would result in a genotype carrying the two versions of the target gene (the SOE version and the WT version) along with the backbone of the suicide vector. This is termed as a single crossover event (SCO). The full deletion version occurs in a double crossover event which is obtained by using the counter selection marker SacB in presence of sucrose. A toxic sucrose by-product would eliminate bacteria that still carry the backbone of the vector (a by-product of the protein levansucrase from the *sacB* gene which is on the backbone of the plasmid). This selects for double crossover (DCO) mutants which lost the backbone of the plasmid but retained the version of the gene with antibiotic marker replacing the coding sequence for the target area (KpGI-2).

Colonies from the sucrose plate were patched on LA-30 µl/ml Apramycin and LA 30 µl/ml Chloramphenicol to screen for the phenotype expected on the DCO (Apramycin

Resistant, Chloramphenicol-sensitive) which would also be indicative of the loss of the backbone of the suicide vector DNA from the chromosome. The aforementioned phenotype could point to a possible DCO event, which was then confirmed by PCR using primers designed to lay outside the flanking region. In a mutant, PCR results in the expected size band that differs in size from the wild-type band.

3- Flippase mediated recombination[106, 115, 116]

All Antibiotic cassettes used in this study were flanked by Flippase Recognition Target sequences, to allow for a recombination effect mediated by Flippase enzyme to remove the antibiotic marker that was present between these two recognition sites from the mutant prior to any assays that involve these mutants. A plasmid containing the gene encoding for Flippase is transformed in the mutants which contain markers pFLP-2-Hph plasmid or pFLP-2_APra. The Flippase is under an IPTG promoter and as mentioned previously, competent cells are prepared after transformation with pFLP-2 plasmids. Colonies that exhibited loss of resistance to the antibiotic marker (detected by patching on LA plates with and without the antibiotic) were confirmed for the expected genotype with colony PCR.

4- Point mutation of the *fic-rl* gene

A mutant sequence of the *fic-rl* gene was constructed in order to be used for cloning into the eukaryotic expression plasmids, as well as into the protein expression plasmids (see below). The conserved histidine in the conserved FIC motif was identified; two base pair changes were introduced to turn the codon sequence for the histidine (a Polar amino acid) into the non-polar Alanine. These point mutations were simply introduced into primers overlapping the sequence upstream and downstream from the sequence coding for the new point mutations. These primers were paired with primers that anneal at the beginning and the end fragment of the gene. PCR with these primers resulted in two fragments each carrying a homologous complementary sequence that carried the desired point mutations; these two fragments were joined by SOE-PCR to create the mutant variant of the gene. This was later verified by sequencing.

2.2.6 Plasmid Construction

Conventional restriction site cloning was used for the majority of the plasmids constructed in this report.

2.2.6.1 Restriction Enzyme digestion and Ligation using T4 Ligase

All restriction enzymes were obtained from New England Biolabs. Digestion was carried out in 25-50µl samples. The buffer was adjusted to a 1X final concentration with the addition of 1 µl of each restriction enzyme stock that contained 20,000 units /ml. The amount of DNA digested was usually >1µg (plasmid DNA). Reactions were performed for 30-60 minutes at 37^{o} C before stopping the reaction with heat inactivation (65-80^{o} C depending on which enzyme used). The resulting DNA was analysed by electrophoresis to verify successful cutting.

For Ligation, T4 DNA ligase was used (Promega), prior to ligation the insert and vector DNA were purified using gel extraction kits, and the DNA samples were concentrated to small volumes (5-10 µl) using vacuum drying. Usually a 3:1 insert to vector ratio is used in the reaction (depends on the size of the insert relative to the size of the vector) to minimize self-religation products. A 10 µl final volume reaction was achieved after adding the ATP containing buffer to a 1X concentration and 3 units of the T4 DNA ligase. The reaction was run at 4^{o} C overnight and then left at room temperature for 1 hour the following morning before transformation.

2.2.6.2 Cloning procedure

Plasmids pWSK29-Apra, pRK6-myc and pGEX-4T-1 Plasmids:

Plasmid pWSK29-Apra was used as an expression plasmid in *Klebsiella* hosts in previously published work [117], and thus it was selected to study the protein secretion and expression of the five genes encoding for Fic proteins in *Klebsiella*. Plasmid pRK5-myc is also a vector used in many studies for transfection purposes and is a potent eukaryotic expression vector, and thus it was used for the transfection and immunohistochemistry assays [118] Plasmid pGEX-4T-1 was used for protein purification studies as it allowed producing GST tagged version of proteins.

Plasmid Editor Software (ApE) was used for in-silico planning of each vector construction and primer design. ApE has many functions, including a selection of restriction enzymes

(specifically those that do not cut the insert but are present in the MCS); viewing of the orientation of the gene once inserted in the MCS, and verification that it is in frame to the start codon in relation to its promoter region.

Many variants of pWSK-29-Apra have been constructed (carrying different inserts of the genes coding for Fic domain bearing proteins in its MCS). Regularly an eGFP carrying vector is constructed alongside these vectors used to ensure correct protein expression (by simple means of exposing the colonies to UV light and detecting fluorescence). The MCS of pWSK29-Apra plasmids lies within the tail end of the *lacZ* gene (which in turn is under an Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible promoter). Using the same restriction sites for all the inserts introduced in each of the 6 variants while remaining in frame with the *lacZ* gene. In order to detect proteins produced by these plasmids, a FLAG TAG sequence was also added to the reverse primers (C-terminus of the resultant protein).

pGEX-4T-1 vectors were constructed similarly; however, they contain the sequence encoding Glutathione S-transferase (GST) upstream of the MCS, and thus any insert added into the MCS would have its start codon removed (as to use the natural start codon of the GST gene) as it is also under an IPTG promoter.

Plasmid pRK5myc used is a high copy number plasmid with a multiple cloning site and a strong CMV promoter. It also contains a myc tag encoding sequence upstream of the MCS that would be added to gene sequences introduced downstream of this tag. Similarly, to pGEX vectors it allows for relatively easy and quick cloning of genes while simultaneously adding a tag to the 5' sequence. The five variants of the Fic domain encoding genes were constructed using BamHI on forward primers and EcoRI on reverse primers, as well as the mutant insert of the *fic-rl* gene.

2.3 Bioinformatics:

2.3.1 Homolog Search using HMMER-3

Basic research tools utilized include Blastn, Blastp, Primer3Plus, Megablast, Blastx, Plasmid editor, clustalW and MUSCLE alignment tool while more advanced tools relying

on UNIX operating system were utilized for homolog search and phylogeny tree construction such as HMMER-3[38] and clustalX 2.0.

HMMER-3 software is an advanced homolog search tool owing to the more complex mathematical models that are superior to BLAST searching in terms of accuracy and ability to detect homologs (as evidenced in this report). HMMER-3 software employs a probabilistic model obtained through the pfam database [39] (called the Hidden Markov Model). The model in the context of a UNIX operating system is able to search locally downloaded databases constructed from online databases such as NCBI genome, protein and gene databases along with pfam.

Each hit is later confirmed by BLASTn and BLASTp and cross-referenced across all sequenced strain on the NCBI database. The FASTA sequence of the nucleotide and the amino acid sequences arre collected and aligned (MUSCLE) for phylogenetic analysis tools (CLUSTALX 2.0).

2.3.2 Phylogenetic Analysis

Multiple Sequence Alignment (MSA) files using MUSCLE were prepared in FASTA input format for clustalX2.0 to obtain a phylogenetic analysis.

Phylogenetic tree construction is based on the inferred evolutionary connection between multiple inputs according to similarities and differences in their characteristics or sequence, which implies their relationship according to an inferred common ancestor.

Rooted trees by neighbour joining (algorithm based on the number of different sequences between different members in the alignment) were obtained and bootstrapped to a value of 1000 (to give a value to the confidence of each inferred node in the tree, similar to p-value in statistics methods, however a value of 1000 between members of a node infers that the probability of error is close to zero and vice versa).

MSA sequences were used to identify conserved sequences in each of the homologs; these conserved sequences were in turn used to design primers to detect each of those homologs using PCR in any given strain.

2.4 Growth and Cell Viability

Mutants that were constructed in this study underwent growth and cell viability assessment using two methods; spectrophotometry using plate reader that assessed bacterial cell density and conventional colony forming unit (CFU) counting.

2.4.1 Growth Curves using Plate Reader

Prior to growing the bacteria, the software on the Varioskan LUX (Thermo scientific) plate reader was calibrated to receive the samples in the following settings:
Blank measurement through the experiment is obtained by assigning wells with fresh medium as a blank; the temperature is set at 37 ^{o} C and the shaking condition at 200 RPM; reading points are set for a reading every 10 minute intervals for a 24-hour period (144 readings), and reading wavelength is set at OD600 nm.
A fresh culture of bacteria on LA plates was grown overnight at 37^{o} C. The following morning a 1/100 dilution was made and the bacteria were allowed to grow to an absorbance at OD600 of 0.2.
From each flask, two rows of wells of the 96 wells plate (Nunc) were filled with equal volumes of 500 µl in each well for the plate reader.
Data was collected and transferred to an excel sheet and then analysed using Graphpad Prism software.

2.4.2 Cell Viability Assessment of Bacterial Growth by Measuring CFU

The bacterial cultures for this step were prepared in a similar manner to the previous step. Bacteria in the exponential phase (as measured by an OD600nm of 0.2 OD) were serially diluted (from neat to 10^{-6}) and plated on LA plates to determine the CFU count for each serial dilution. The purpose of this experiment is to assess the cell viability of the same colonies that were assessed using growth curves above.

After overnight growth at 37^{o} C plates were collected and the colonies counted. For optimal counting, plates with 30-300 colonies were selected and the calculation factors in the dilution of the bacteria plated as well as the volume were taken into account. A final number of CFU/ml was obtained (from plating in triplicates). The average of the three numbers were recorded as the CFU/ml for that time point. The flasks were then allowed to grow at the same conditions as the plate in the varioskan plate reader, and a CFU count measurement was repeated at different time points in the same method, (at 3 hours, 6 hours, 9 hours, 12 hours, 16 hours, 24 hours) the data was collected and analyzed using Graphpad Prism.

2.5 Biofilm Formation Assay

The method employed in this study was essentially described in [119] which involved three steps:

2.5.1 Allowing Bacteria to Form and Establish Biofilm

A single colony was picked from a freshly grown bacterial culture from a single colony of the required strain. For this experiment, the wild-type HS04160 and the 4 variant Fic domain mutants were used.

A fresh bacterial colony was inoculated in LB and grown overnight at 37^{o} C. The following day, a 1 in 100 dilution was made, and the bacteria were allowed to grow to an absorbance at OD600 nm of 0.2. This culture was then used to make serial dilutions, which were put into 96 well plates (Nunc) similar to the method used in growth curve formation (a 100 µl volume was used in each well).

Finally, identical plates were incubated overnight at 37^{o} C and 30^{o} C to measure biofilm formation at these two temperatures.

2.5.2 Biofilm Staining:

After the incubation period, any un-detached cells were mechanically removed by shaking the plates and discarding out the unbound bacteria. This was then followed by a careful flooding of the wells in a container filled with distilled water. Flooded wells were emptied by shaking and dumping out the water and this process is repeated twice. The actual staining procedure involved adding 100 µl volume 0.8% crystal violet solution into each well. An incubation period of 30 minutes was followed by a washing step using the same water container from the method mentioned above, this was repeated 3-4

times to wash out the excess stain. The plates were then placed on a tissue paper with occasional blotting and allowed to dry overnight.

2.5.3 Biofilm Solubilising and Quantifying:

125ul of 70% ethanol was added to each well and, incubated for 30 minutes with occasional gentle shaking. The plate was then transferred and read using a varioskan plate reader at an absorbance of 600nm, with a few wells used as blank and filled with only 70% ethanol. The wild-type strain was used as reference point to the mutants tested.

2.6 Virulence Assay Using *Galleria mellonella* Larvae

The methods for this assay were done by following protocols published in [120, 121] with minor modifications.

2.6.1 Insect Preparation:

The insects were purchased from LiveFoods UK. The larvae usually arrived within 3 days of ordering and were kept at 25^{o} C in the dark. The larvae were stored in plastic containers which permit air exchange with a padding of wood shavings inserted in the tubs. Handling the insects was done with blunt instrumentation [120]. Insects were selected first according to size (2-3 cm) and weight (200-250mg), which corresponds to the 6^{th} stage of their life cycle. At the 6^{th} stage of life the larvae remain for roughly two weeks without the need of nutrients before pupating. Any larvae showing signs of inactivity or pupating (hard darkened exterior) were discarded

For each experiment, a replicate group of 15 larvae has an additional 3 larvae for control purposes. The larvae were split in Petri dishes one day before injection.

2.6.2 Inoculum Preparation:

From an overnight culture of appropriately selected bacteria, a 1 in 100 dilution was obtained, the bacteria were allowed to grow for 3-4 hours. The bacteria were then pelleted and washed 3 times with fresh sterile and filtered PBS. The cells were then resuspended in this PBS and adjusted to an absorbance of 0.2 at OD600nm. At this point, five-time serial dilutions of the bacteria were obtained in PBS. The five dilutions are

made by a factor of 10 (neat, 1/10, 1/100/ 1/1000/ 1/10000). For each dilution, a replicate vial was made and 100 µl of the sample was plated on LA plates for CFU counting. In addition, a vial of the same PBS used was left aside to be injected as a control.

Injection of the larvae was done by using a specialized low volume injector (capable of consistently injecting small volumes) and an insulin syringe (31 gauge BD syringes). A volume of 10 µl of each sample or the PBS control was injected into the bottom left pro-leg into the haemocyle.

2.6.3 Analysis of Infection Results:

Following injection, the larvae were incubated in their plates in a 37^{o} C incubator and their mortality was assessed every 24 hours. Insects were examined daily with a blunt object as to minimalize injury and were gently flipped on their backs or prodded to check for signs of movement. Live larvae are quick to flip back but sickly larvae that are still alive are slow and show dark skin pigmentation and usually die in the following days. Each group was assessed every 24 hours, and the CFU count was noted for each group, the experiment was allowed to run for 5 days.

This process was repeated three times for each of the mutant strains; however, before testing the mutants, a baseline for the wild type had to be established. The killing assay was first performed several times on the wild type as described above, and the concentration of bacteria that caused the death of half of the population of each group (7-8 larvae in a 15 larvae group) at the end of the 5-day period (LD50) was established. After establishing a consistent method and LD50 for the wild type strain, each of the mutants was assessed following the same process with the same doses, and results were noted.

2.7 Protein related techniques

Table 2-4 Materials used in this section

Buffer	Components
SDS-PAGE gel-staining solution	0.25%(W/v) Coomassie Brilliant Blue R250, in 25% methanol and 10% v/v glacial acetic acid in distilled water
SDS-PAGE gel destaining solution	25% ethanol, 10% acetic acid in distilled water
SDS-PAGE running buffer (10X solution dilute to 1X for use) pH8.3	30.3g Tris base, 144.1 Glycine 50ml 20g SDS topped up to 1L with distilled water
Protein sample loading dye (buffer)	10%SDS w/v, 20% Glycerol (v/v) 5% mercaptoethanol , 0.2M Tris –HCL pH6.8 and 0.05% w/v bromophenol blue
Western blot transfer buffer pH8.3	5,6 g Tris base, 2.9g Glycine, 0.37 g SDS, 200 ml Methanol topped up to 1L with distilled water
Tris Saline 10X	0.1M Tris, 1.5M NaCl in distilled water (pH 7.4)
Western Blot wash buffer (TST 1X)	10% (v/v) of 10X Tris Saline and 0.1% (v/v) Tween-20 in 1L distilled water
Blocking buffer	5% w/v skimmed milk in TST
SDS-PAGE resolving gel	12% gel = 4.9ml of H2O, 6ml of 30% (acrylamide-Bisacrylamide Protogel) 3.8 ml of 1.5M Tris (pH8.8), 150ul of 10% w/v SDS,150ul of 10% w/v Ammonium Persulphate with the addition of 10 µl TEMED (Tetra methyl ethyl enediamine) just before gel pouring
SDS-PAGE stacking gel	4.1ml of H2O, 1ml of 30% (acrylamide-Bisacrylamide Protogel) 750ul of 1.0 M Tris (pH6.8), 60ul of 10% w/v SDS,60ul of 10% w/v Ammonium Persulphate with the addition of 10 µl TEMED just before gel pouring

2.7.1 SDS-PAGE:

Gel casts and electrophoresis equipment (Biorad) were used for these experiments; the gel was made as shown in table 2.4

50 µl of protein samples to be run were mixed with 5 µl of 5X SDS loading dye (1x concentration) and heated for 5 minutes at 100^{o} C prior to loading onto gel lanes. A protein standard marker was used for each run. Gels were usually run at 150V for an hour and 10 minutes. As the dye part reaches the end of the gel cast, the electrophoresis was stopped and gels were transferred to a hard-plastic container and covered with staining solution (Coomassie blue). The gels were allowed to stain for 2-3 hours until the gel was no longer transparent and appeared solidly blue. Gels were destained by covering the gel with destaining solution and gently shaking until bands were clear blue and the background was clear.

SDS-PAGE images were obtained using a Biorad image scanner.

2.7.2 Western-Blot (Wet Transfer)

Western blotting was performed to transfer the protein bands separated by an SDS-PAGE to a nitrocellulose membrane (Hybond-C super-GE lifesciences). After the SDS-PAGE step, the gel was soaked in transfer buffer for 15 minutes (nitrocellulose membrane and Whatman 3mm sheets and sponges used for cast assembly were also soaked in transfer buffer). The transfer was done using Western blot apparatus (Biorad) in a 4^{o} C cold room or with the addition of ice packs/gels to the transfer tub to control temperature

Western blots were assembled as follows: from the negative electrode (or clear/red coloured) part of the cast:

<sponge-Whatman paper-Nitrocellulose Membrane-SDS gel-Whatman paper-sponge>.

The transfer was allowed to run at 150 mA for 70 minutes, following which the membrane was incubated at room temperature with blocking buffer for 1 hour to overnight. The membrane was then washed three times (10 minute wash for each step while gently shaking) with wash buffer, and then a primary antibody solution (1/500 dilution) in blocking buffer was added to the washed blot and allowed to incubate for 2-3 hours at room temperate or at 4 ^{o} C overnight. For non-conjugated antibodies, a secondary antibody was added (1/1000 dilution) after washing away the primary

antibody. The membrane was incubated for 1 hour. Membranes were washed 3 times as previously described and processed for immune detection.

2.7.2 Immune detection:

Chemiluminescence ECL Kit (PIERCE) or similar horseradish peroxidase based kits were used at this step. The detection reagents were prepared as per manufacturer's instructions and added on the marked protein side of the membrane. Following incubation for the recommended 2-minute period; the membrane wa covered with clear, thin plastic wrap and excess detection reagent was gently removed with tissue paper.

The detection was then performed in a dark room where the membrane with the substrate added was exposed to an Amersham Hyperfilm autoradiography film and allowed exposure for 1 to 5 minutes.

The films were developed using developer and fixer solutions provided by AGFA. The films were washed with distilled water and allowed to dry before taking a photograph or digital scans of the resulting image.

2.8 Secretion Assay

2.8.1 Bacterial Strains Used for Expression and Secretion Experiments

Strains that were assessed for their ability to secrete Fic domain bearing proteins were first transformed with pWSK29 plasmids containing inserts encoding each of the Fic proteins tagged with the FLAG tag as described in the cloning section. Each strain was confirmed to contain the correct plasmid using colony PCR (using primers that lay on the plasmid backbone outside the MCS) and the appropriate antibiotic selection. Bacterial strains were then grown at 25^{o} C overnight with shaking at 200RPM with the appropriate antibiotic.

2.8.2 Temperature Shift

The overnight cultures were diluted to a 1 in 100 subculture, and the bacteria were allowed to grow for five hours in a 100 ml LB broth at 37^{o} C with the addition of antibiotic (30 µl/ml Apramycin) and inducing agent (IPTG) to a concentration of 50 µM. This process results in a majority of bacteria to be in the log phase when entering the temperature shift, which minimises autolysis and unspecific release of intracellular

proteins into the supernatant. This helps to avoid false positive results (autolysis occurs when bacteria are put in stressful conditions such as exposure to antibiotics, or osmotic pressure). [122]

2.8.3 Autolysis Measurement:

Although practices were adopted to minimise the effect of autolysis on the outcome of this experiment, a simple assessment of the autolytic activity happening in the subculture at 25^{o} C and 37^{o} C was performed at 3 different time points (1 hour, 3 hours and 6 hours). This was done by measuring the DNA content in the supernatant at these time points relative to fresh medium as a blank using nanodrop for measurements.

2.8.4 Protein Collection

The protein expression and induction were tested to verify the optimal time point for the expression and secretion. After the temperature shift incubation, bacterial samples were pelleted and the supernatant was collected and tested for the presence of secreted proteins. The remaining pellet was used to verify the presence of the expressed proteins located intracellularly.

To concentrate proteins, Strataclean resin (Agilenet) was added to the collected supernatant. This resin unselectively binds proteins, thus allowing them to be pelleted [123]. 50 µl of the resin was added to the supernatant, and the samples were occasionally mixed on ice for 10 minutes. This was followed by centrifugation at 4400g for 15 minutes at 4^{o} C. The supernatant was then removed and the pellet was resuspended in 1 ml of the medium and then transferred to a sterile Eppendorf tube. The tube was then spun down at 14000 G, and the supernatant was drawn off. The resin pellet samples were heated for 5 minutes at 100 ^{o} C with SDS loading dye to release the protein from the resin (this would represent the secreted fraction of the proteins).

Protein extraction from the bacterial pellets was done similarly. Pellets were resuspended in 150 µl solubilizing buffer (20mM Phosphate buffer pH8.0, 300mM NaCl 2% w/v SDS, 2mM dithiothreitol, 1x protease inhibitor cocktail and 1%v/v Triton X-100), and the cells were lysed by sonication (sonication on ice, 10 second pulses for 1 minute rest for 10 cycles), the samples were spun down at 10000g for 30 minutes at room

temperature and then supernatant was drawn off (this would represent the intracellular protein fraction).

2.8.5 Protein Detection

Samples from both fractions (secreted fraction, intracellular fraction) were subjected to SDS-PAGE and Western blotting for detection of the FLAG-tagged FIC domain bearing proteins.

2.9 Immunohistochemistry

2.9.1 Tissue Culturing and Cell Line Maintenance

Two cell lines were utilised for this section of the project, HK-2 cell line (Human Kidney-2), an immortalised adult proximal tubular epithelial cell line [124] and A549 human epithelial lung carcinoma [125]. Basic cultivation and maintenance work related to this part of the project was done in specialised class 2 safety cabinets under strict aseptic techniques.

Cell lines were obtained as liquid nitrogen frozen vials in 10% v/v DMSO (Dimethyl sulfoxide) in a serum free medium (also used for cryopreservation). Retrieved vials were incubated in a 37^{o} C water bath until thawed, then were centrifuged at 1000g for 5 minutes. The cell pellet was transferred to an appropriate size tissue culture flask (Nunclon delta, 75 cm^2 culture surface).

The growth medium used was Dulbecco's modified eagle F12 medium with Glutamax (DMEM/F12) (Thermofischer) supplemented with 10%(v/v) heat inactivated tissue culture grade Foetal Calf Serum (FCS) –(Thermofischer) (50 ml in 500 ml bottles) and 1X Penicillin-streptomycin antibiotics (5ml in 500ml bottles) (Sigma), which were all filter sterilized prior to addition to the stock medium.

13 ml of Growth medium was added to each flask and cells were grown in 37 ^{o} C 5% CO_2 controlled incubators. Growth medium was changed at regular intervals of 3-5 days (according to the phenol red indicator and published recommendations) and the cells were split for a maximum of two times. Cell splitting was done using Trypsin (when cells reach near confluence). The growth medium was removed, the trypsin solution (10% v/v) was added and cells incubated for 10 minutes. To inactivate trypsin two equal

volumes of the growth medium was added to the cells. Cells were spun down at 1000g for 10 minutes allowing for the collection of the pellet which was resuspended in growth medium and split into 2 or more flasks.

2.9.2 Transfection:

Tissue culture cells were seeded to around 50-70% confluence on glass coverslips in 24 well plates. Medium was not changed in the 24 hours prior to transfection as to allow cells to be partially starved (improves transfection efficacy). Cells were checked by regular light microscopy prior to transfection to verify healthy cell population on the coverslips. Each well was incubated in 500 µl of medium for growth.

Minimum serum medium (Opti-MEM) was used to mix the transfection reagent Lipofectamine (Thermo Fischer) with plasmid DNA according to manufacturer's instructions. The DNA-Lipofectamine mixture was allowed to incubate for 5 minutes at room temperature, the DNA-lipid complex was then added to each well of the cells in the 24 well plates. The cells were incubated at 37^{o} C overnight (or up to 72 hours in case of HK-2 cells temporal effect study).

2.9.3 Immunohistochemistry and Slide Preparation

Each step in this section was preceded by two washes with Dulbecco's PBS (tissue grade PBS). Following the first wash procedure, a fixative (4% w/v Paraformaldehyde) was added and allowed to incubate at room temperature for 15 minutes. Samples were then permeabilized using 0.5% v/v Triton X-100 for 2 minutes.

Samples were then blocked for 1 hour using 5% w/v BSA in PBS, and the primary anti myc antibodies (Monoclonal Anti c-Myc antibody produced in mouse, clone 9E10, ascites fluid, Sigma-Aldrich) which were diluted in an antibody dilution buffer (1XPBS +0.3% w/v BSA) at 1/1000 ratio were added. The cells were then incubated for 1 hour followed by a three-time wash step.

200 µl of 100nM Rhodamine phalloidin (Cytoskeleton Inc.) was added to each well for cytoskeleton staining and incubated in the dark at room temperature for 1 hour. Lastly, the Alexa Fluor® 488 Donkey anti-Mouse IgG Antibody (life technologies) was added for myc tag protein detection and incubated for 1 hour also at room temperature.

Coverslips were labelled and mounted on glass slides with the addition of a drop DAPI/antifade solution (Vectarshield-cytoskeleton inc.) and sealing the coverslips with clear nail varnish. The mounts were allowed to dry and then visualised using EPI fluorescence and confocal microscopy.

2.10 Enzymatic Activity Assay

2.10.1 Protein Expression

pGEX-4T-1 based plasmids were used for this set of experiments. Two plasmids carrying either the wild-type *fic-rl* gene or the mutant constructed version of *fic-rl* were constructed and verified by colony PCR and sequencing.

The protein expression was done as previously described in section 2.8.2., however, different expression temperatures were tested, and the induction phase was left overnight to maximise protein yield.

After expression at the optimal temperature (17 ^{o} C for Fic-RL and 25 ^{o} C for Mutant Fic-RL) in 500ml LB broth with antibiotics, the samples were pelleted and the pellet frozen in a -80 ^{o} C freezer for two hours/overnight to improve protein yield and inhibit cellular proteases. The pellet was thawed in Binding-Lysis buffer (50 µl of DNase -BBI Solutions-, 5mM $MgCl_2$ and a tablet of protease inhibitor cocktail -Roche- all dissolved in sterile PBS). After thawing the pellet was dissolved in lysis buffer and then sonicated (10 pulses 45 seconds each with a minute rest in between). The lysed pellet was then centrifuged at 15000 G for 20 minutes at 4^{o} C, leaving the soluble protein in the supernatant.

2.10.2 Affinity Chromatography

Protein purification protocol was adapted from Harper et al method[126]. PBS wash was routinely done after each step. Glutathione resin (the affinity molecule binding the GST tag) was purchased from (GE Healthcare Life Sciences). A column based method was used where the resin was added to a purification column and the excess ethanol was allowed to run off. The columns (Thermofischer) were then washed with PBS by running PBS through the column and the supernatant was added to the column and allowed to pass through. GST-tagged protein was bound to the resin in the columns and then eluted out of the resin using a reducing agent present in the elution buffer (5mM

Diotheothrietol,50mM Tris, and 10mM reducing glutathione-Duchefa Biochemie at pH 8.0). Elution was done in 1ml fractions and each fraction was screened for the presence of the protein using SDS-PAGE. Correct size bands were sent for sequencing to PNACL at University of Leicester for confirmation (10-100 µl pipette tip size was taken from each band for sequencing) .

2.10.3 Assessing Rho GTPase activity

To measure the guanine exchange activity, purified Fic-RL and purified Fic-RL mutant proteins were incubated with the three best known Rho GTPases (Rac1, Cdc42 and RhoA), and reagents were provided in the Rho GTPase activity kit (Cytoskeleton Inc). Human Dbs, protein a known strong activator of GTPases, was included as a positive control.

The assay was performed as per manufacturer's instruction, using a fluorophore-modified substrate that can be measured when utilized by a Guanine Exchange Factor (when the Mant-GTP substrate is utilized by a GEF and is bound to the GTPase protein, fluorescence index is significantly increased allowing it to be recorded using a fluorescence plate reader (Varioskan)). The activity of these GTPase was measured in the presence and absence of sample proteins over 30-60 minute periods and compared to the positive control. Equal µM concentrations of proteins was introduced into each well for each reaction (Rho GTPase, GST, Fic-RL or mutant Fic-RL) at a 10:1 ratio (10 for Fic-RL, GST or mutant Fic-RL against 1 of Rho GTPase tested).

Chapter 3

Bioinformatics and Secretion Analysis

3.1 Bioinformatics and Secretion

3.2 Introduction:

For a bacterial toxin that targets intracellular machinery to exert its function, the toxin must first be delivered into its target host cell. Some such toxins are secreted by the bacteria and then taken up by the host cell. The classical examples are the diphtheria toxin [127, 128], cholera toxin and heat labile *E. coli* toxins[129]. However, other bacteria employ their secretion systems such as T4SS [130-132] or T6SS [133] to directly inject toxins inside the eukaryotic cells (or other prokaryotes).

As mentioned in the introduction chapter, *K. pneumoniae* harbours different types of secretion systems, namely T2SS, T4SS and T6SS. Many Fic proteins with toxic effects on host cells have been identified in different bacteria. Their targets within the cell could be Rho or RAB GTPases in eukaryotes or EF-Tu elongation factor in bacteria. Once delivered, these Fic proteins cause deleterious effects on host cell. Table 1.2 depicts some of these Fic proteins and the secretion system that has been shown to secrete them. It is apparent that most of these Fic proteins described in the literature so far are secreted by T4SS and less so by T3SS.

While *K. pneumoniae* does not have a T3SS machinery it does carry operons coding for two T4SS[134]. Both these operons are carried on self-transmissible elements called Integrative and Conjugative Elements (ICE) [135, 136]. These elements in *K. pneumoniae* code for different homologs of the T4SS that aids them in the assembly of this conjugation apparatus and to transmit themselves (and other DNA molecules) horizontally by being injected into neighbouring cells [99, 137]. ICE elements also code for genes that help them integrate into the genomes, once delivered into the target cells, and code for genes that help them mobilise DNA to be delivered by means of conjugation [137-139]. T4SS can also deliver proteins into target cells alongside DNA molecules [49, 140]. While many T4SS subclasses have been revealed, they all contain many homologous proteins amongst themselves that code for vital structures of the

machinery (inner membrane porin, cylindrical apparatus or ATPase). However, the VirB4 ATPase protein seems to be a ubiquitous member of these subclasses[101].

3.3 Aims

To identify Fic domain bearing protein homologs in *K. pneumoniae* and identify which of these proteins are secreted by these bacteria, followed by identification of the secretion system responsible for secreting these proteins.

3.4 Results

3.4.1 Bioinformatics

Basic Bioinformatic research tools including BLASTn, BLASTp, Primer3Plus, MegaBLAST, BLASTx, Plasmid editor, clustalW and MUSCLE alignment tool were used to identify and verify homolog hits, while more advanced tools relying on UNIX operating system were utilised for homolog search and phylogeny tree construction such as HMMER-3[38] and clustalX 2.0.

The basic tools were used to identify the domain in question firstly in the ORF 5 of the KpGI-2 Island to characterise the type of island that some strains of *K. pneumoniae* carried. The examination of KpGI-2 was the primary finding into identifying the Fic domain as a possible tool in the pathogenesis of this bacterium. The online protein family database search tools (pfam) identified the ORF 5 of KpGi-2 (later described as *fic-gn*) as a member of the newly described family of Fic domain bearing proteins.

HMMER-3 software is an advanced homolog search tool, owing to the more complex mathematical models that is superior to BLAST searching in terms of accuracy and ability to detect homologs (as evidenced in this report). HMMER-3 software employs a probabilistic model obtained through the pfam database [39] (called the Hidden Markov Model). The model in the context of a UNIX operating system is able to search locally

downloaded databases constructed from online databases such as NCBI genome, protein and gene databases along with pfam.

The use of this software has enabled the detection of five different genes in *K. pneumoniae* encoding proteins that bear a fic domain (figure 3.1) and that were not detected using Blastn and Blastp at first. Each homolog was later confirmed by BLASTn and BLASTp and cross-referenced across all sequenced strain on the NCBI database (Figure 3.2). The FASTA sequence of the nucleotide and the amino acid sequences was collected and aligned (MUSCLE) for phylogenetic analysis tools (CLUSTALX 2.0) (see next section).

Three out of the five homologs (*fic-ad, fic-rl* and *docKp*) were present in the same location on the genomes of the 80+ fully sequenced *K. pneumoniae* strains on the NCBI database (these fic genes were flanked by the same genes on both sides). However, *fic-gn* was the only homolog to be present on a horizontal gene transfer element (it is present in KpGI-2 genomic island) as shown in figure 3.2 and in the next section.

3.4.1.1 Phylogenetic Analysis

Multiple Sequence Alignment (MSA) files using MUSCLE were prepared in FASTA input format for clustalX2.0 to obtain phylogenetic analysis, as mentioned in the methods section, for the five homologs of Fic proteins. Rooted trees by neighbor-joining (algorithm based on the number of different sequences between different members in the alignment) were obtained and bootstrapped to a value of 1000 (to give a value to the confidence of each inferred node in the tree, similar to p-value in statistics methods; however, a value of 1000 between members of a node infers that the probability of error is close to zero and vice versa).

Multiple sequence alignments of nucleotide sequences of fic genes taken from the first available fully sequenced strains of *K. pneumonia* were obtained and used to identify conserved sequences in each of the homologs. These conserved sequences were, in

turn, used to design primers to detect each of those homologs using PCR in any given strain (which were used as mentioned in Figure 3.1.A to confirm database searches using PCR as well as confirm gene deletions in subsequent chapters). The five homologs identified were then subsequently used to determine phylogenic relationship shown in figure 3.1 B.

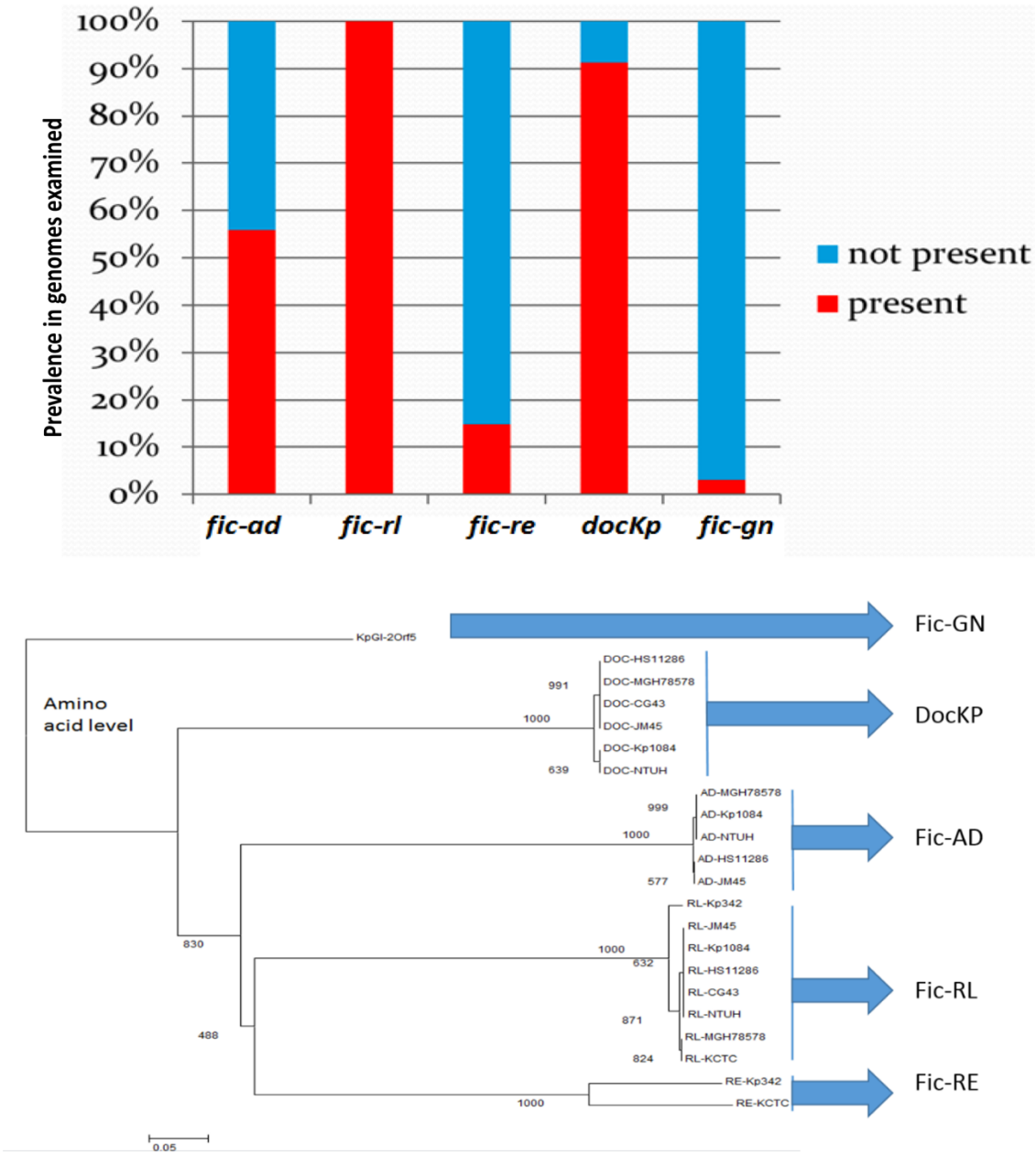


Figure 3-1 A. prevalence and distribution of the fic genes homologs in *Klebsiella pneumoniae* as shown in a total on 49 fully sequenced strains available on NCBI database. Confirmatory data using a small collection of strains showed a similar distribution (especially FIC-RL) being present in all tested samples using primers constructed using multiple sequence alignments of these genes (3.1 B) phylogeny tree at the amino acid level of the 5 FIC homologs in 8 reference strains of *K. pneumoniae*, bootstrap replicates of 1000 were used, alignment done in MUSCLE and tree constructed using ClustalX.

3.4.2 Genes coding for Fic domain bearing proteins

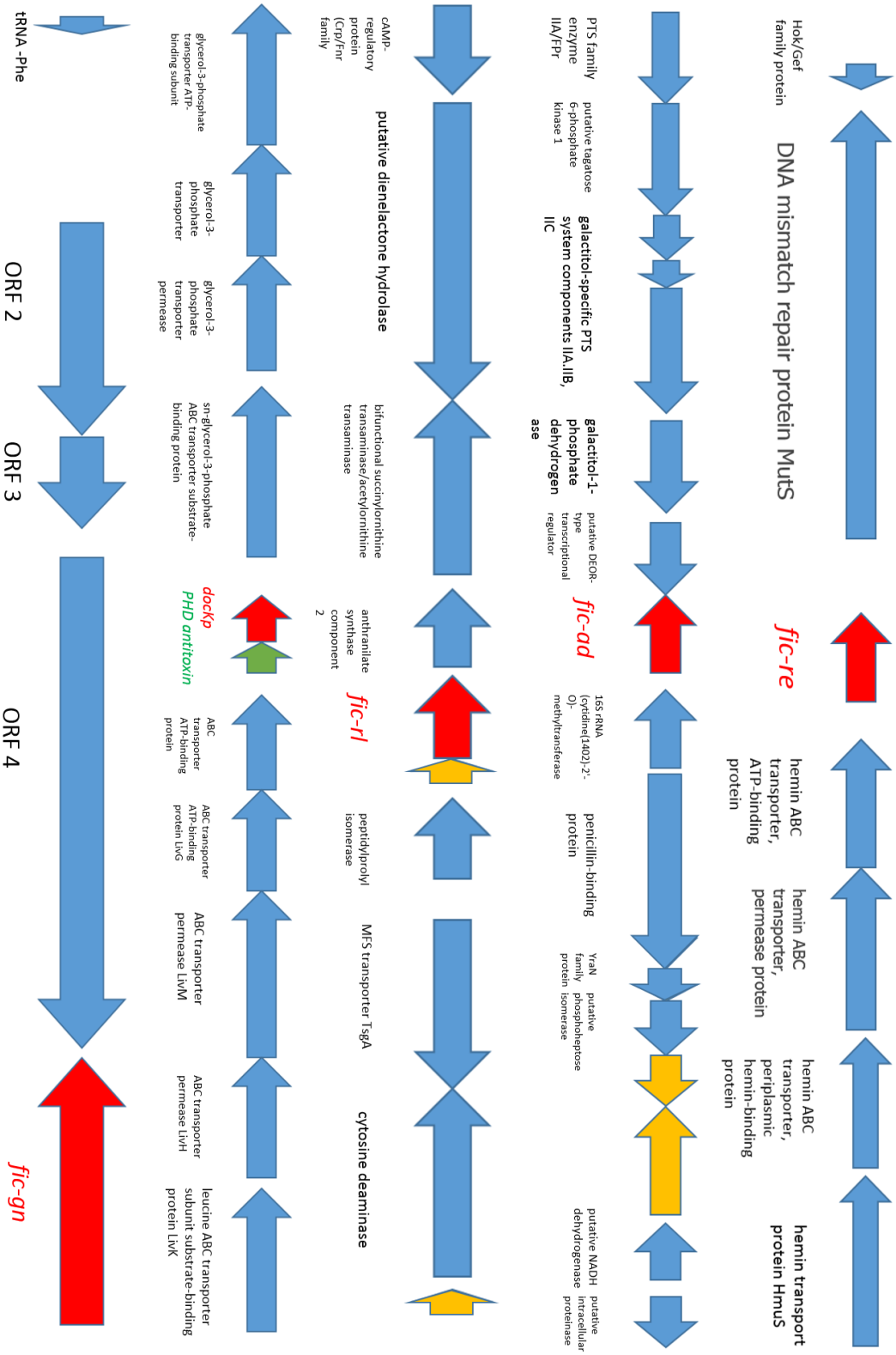


Figure 3-2. Genetic maps of the five genes coding for Fic domain bearing proteins in *Klebsiella pneumoniae* obtained from the NCBI database. The arrows indicate reading frames and the direction of the arrows indicate the orientation of the reading frame (forward vs. reverse in context to surrounding genes), the genes indicated by red arrows are the "fic genes", yellow arrows indicate unannotated genes coding for hypothetical proteins. Arrows in blue indicate genes that have been annotated in the database, with each gene annotation written below each gene. The green arrows indicate the gene coding for the antitoxin part of the Doc/PHD toxin antitoxin system.

Pooling the information gathered on these genes from the above Bioinformatic analysis, a few points on each gene can be summarised as shown in table 3.1.

- As for the first gene *fic-ad,* this gene is present in about 55% of *K. pneumoniae* isolates sequenced and present in the NCBI database (58 strains). Analysis of the gene context reveals that the gene is mainly surrounded by genes annotated as metabolism genes for sugars/sugar alcohol products. Upstream from this gene, a gene coding for penicillin binding protein is found as well as the 16sRNA for methylation of cytidine.

-Gene *fic-rl* is the only one of the five genes conserved in all sequenced strains in the NCBI database. Genetic context reveals genes coding for enzymes involved in DNA synthesis and metabolism as well as a cyclic AMP regulatory protein downstream of *fic-rl* gene.

-As for other Doc/PHD toxin-antitoxin systems, both the components are coded next to each other on the genome of approximately 90% of the strains present in the NCBI database. PHD (Prevent Host Death) gene is coded upstream of the toxin Doc (Death on Curing). Many elements of the ABC transporter system are present upstream of these

genes, indicating perhaps that this mechanism is involved in the transfer of the toxin/antitoxins to other hosts. Glycerol metabolism genes are present downstream of the *doc*/*phd* genes.

-The gene *fic-re* was only present in 4 strains out of the 58 sequenced strains, however, it is present in the same location and gene context is the same for those 4 strains, indicating that this gene may be an acquired element that inserts to the same location. As is the case for the gene coding for the Doc toxin, *fic-re* is surrounded by genes coding for elements of the ABC transport system, perhaps indicating that this gene is associated with this transport mechanism.

-Genomic Island KpGI-2 was present in *K. pneumoniae* isolate from a urine sample. The island hosted one of the five fic homologs as the fifth open reading frame in this island. KpGI-2 a 6.3 kb island, codes for five proteins one of which is a truncated phage-like integrase as well as two helicase-like proteins with *fic-gn* as the last open reading frame. Blast search of this gene reveals it is present in only one isolate of *K. pneumoniae* present on the database, however, the gene is shown in many *Salmonella enterica* isolates with 94% similarity of 100% cover query of *fic-gn*. This would not be surprising when considering that this gene is present on a horizontal genetic transfer element, and indicates likely of the origin of this gene.

Table 3-1 shows a summary of information gathered on the proteins coded by the five genes coding for Fic proteins in *Klebsiella pneumoniae*. With each coding gene and the corresponding protein length estimated produced from the gene. The conserved Fic domain in each homolog is indicated. *docKp* did not adhere to the Fic motif paradigm however it was indicated as a Fic domain by phylogenetic and HMMER-3 analysis

Gene	Protein length	Estimated size (kDa)	Conserved domain
fic-ad	333	33 kDa	HPFADGNG
fic-rl	200	23 kDa	HPFRLGNG
fic-re	205	21 kDa	HPFREGNG
docKp	122	13.5 kDa	
fic-gn	380	43 kDa	HPFGNGNG

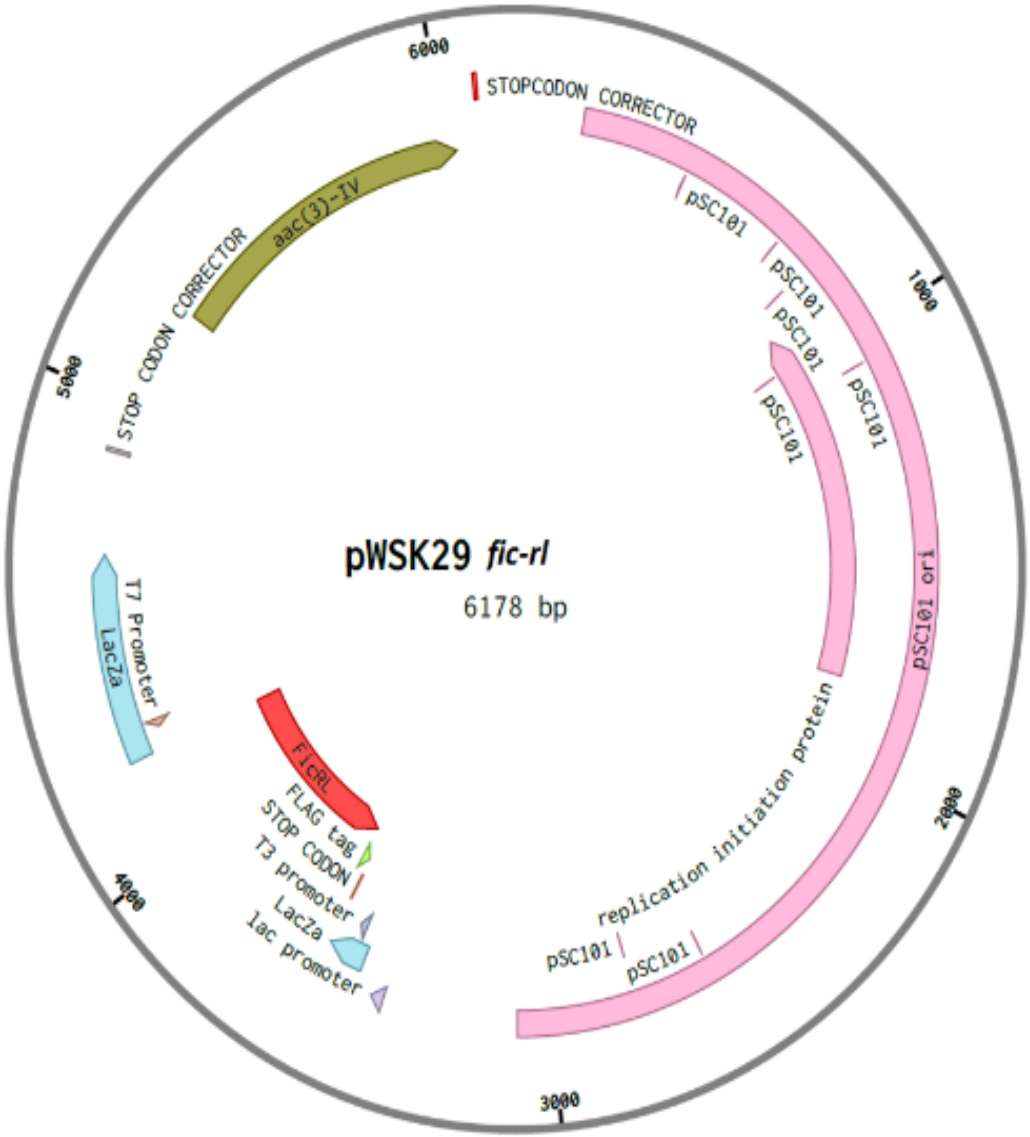
3.4.3 Secretion Assay

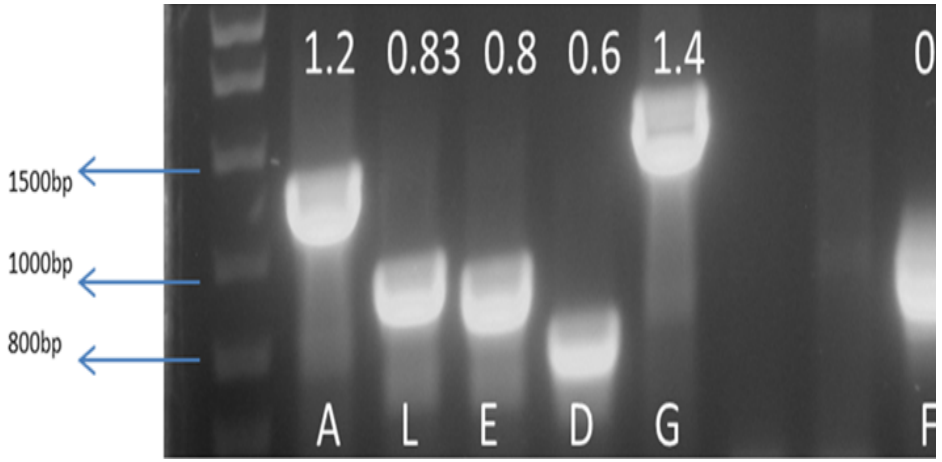
As mentioned in the introduction to this chapter, most (if not all) Fic domain bearing proteins that are toxic in nature are delivered by means of specialised secretion systems (T3SS, T4SS mostly) to induce their action on the host cell. To determine whether any of the Fic proteins in *K. pneumoniae* is secreted by the bacteria a secretion assay was performed. Although secreted proteins are possibly toxic in nature, this assay would also help point out which secretion system is responsible for the secretion of any potential effector.

3.4.3.1 Plasmid construction.

Genes coding for the five Fic homologs in *K. pneumoniae* were cloned into plasmids that would enable the expression of each these proteins tagged with a FLAG on the C-terminus of the protein. As described in the cloning section the tag is added by means of primers. The genes were amplified from chromosomal DNA extracted from strain HS11286, H04160 or Kp342 (as no one strain has all 5 of these genes). Plasmid pWSK29-Apra, a modified version of the plasmid pWSK29, which has been previously used in the expression of proteins in *K. pneumoniae,* was used as the expression vector [117, 141].

These tagged proteins were then used to determine whether they are secreted or not, and which secretion system is responsible for their secretion as shown in figure 3.3. The expression and secretion of these proteins were detected using Western Blot techniques. The plasmids were transformed in the wild-type background strains and mutants lacking certain components of secretions system for assessment as shown in Table 3.2.


6000
STOPCODON CORRECTOR
pSC101
pSC101
pSC101
pSC101
pSC101
pSC101
1000
5000
STOP CODON CORRECTOR
aac(3)-IV
pWSK29 fic-rl
6178 bp
T7 Promoter
LacZa
FicRL
FLAG tag
STOP CODON
T3 promoter
LacZa
lac promoter
replication initiation protein
pSC101 ori
pSC101
pSC101
2000
4000
3000


1.2
0.83
0.8
0.6
1.4
0
1500bp
1000bp
800bp
A
L
E
D
G
F

pNSK29
lac promoter
M13 primer reverse
LacZa
T3 promoter
FLAG tag
FlcRL
T7 Promoter
M13 primer forward

Figure 3-3 (A) plasmid map of the pWSK29-Apra- based plasmid that was constructed with each of the *fic-rl* gene inserted into the MCS using the restriction sites that were selected for the cut and ligate cloning procedure with the corresponding enzymes that do not cut the original sequence of the insert to be introduced as described in methods section gel image showing the PCR confirmation of the 6 clones similarly cloned like the *fic-rl* clone with each different Fic homolog inserted in the same area as *fic-rl* shown in the figure. Clones where verified using the M13 primers that lie outside the insert and MCS, lanes beginning from right of the ladder are as follows : (A)= *fic-ad,(L)= fic-rl,(E)= fic-re,(D)= docKp,(G)= fic-gn* and(F)= *egfp* at the far right , note there is roughly a 227bp increase to the original size of each insert due to the regions outside the MCS incorporated with the M13 primers as shown in the sequence in 3.3.B. Expected size bands are shown in estimates in kb on top of each lane, each band was confirmed by sequencing. In (3.3.B) DNA sequence is shown for that area (in one of the 6 pWSK29 clones, pWSK29 *fic-rl*) , the corresponding area in the plasmid map is roughly shown to indicate the area of the DNA sequence shown. Annotation for each feature of the sequence, the location of the M13 primers is shown in the sequence with adjoining areas. The area shown highlights the cloning method with promoters for each gene as well as the location of the FLAG tag. Sequencing results are aligned with the sequence above to verify each construct, the same procedure is done similarly for the rest of the constructs.

To assess if any of the Fic proteins were secreted, bacterial cells transformed with each of pWSK29/Fic constructs were cultured at room temperature first (in temperature shift method to induce secretion, bacteria are cultured at the temperature that they are exposed to in the environment before shifting them to a host body temperature to simulate entry into host which usually triggers virulence genes at this point as indicated by RNA expression of virulence genes[142]) and then exposed to an immediate stimulus that partially resembles their arrival into a host tissue. This such stimulus could potentially be temperature shifting, additions of certain known cell factors, or simply being adjacent to living host cells.

Previous experimentation with other bacteria and secretion assays (such as *E. coli*, *Burkholderia*) [118, 143] employed the temperature shift model to induce secretion. This was adopted in this project. Secreted proteins were collected from the supernatant of the cultured bacterial cells. The intracellular fraction was also analysed as described in the methods section.

Table 3-2 shows the strains (and their description) that were used to assess the secretion of Fic-RL protein. The other 4 Fic homologs showed no secretion and were not conserved genes as was *fic-rl* and thus Fic-RL secretion was chosen as the focus point.

Strain	*Description of the strain*	*Fic-RL Secretion*	*Reference*
HS11286 ΔMDR	Strain HS 11286 modified with the Multi Drug Resistance island removed for safety reasons	YES	MDR region identified and deleted by Dexi Bi et. al.[144]
HS11286 ΔMDR: ΔT6SS-1: ΔT6SS-3	Strain HS11286 ΔMDR with inframe deletion of locus 1 and locus 3 of the Type 6 Secretion system rendering the strain devoid of the machinery for this secretion system	YES	Mutation constructed by David Ngmenterebo, University of Leicester Lab212 in III Dept. clusters of gene identified in Sarris e.t al.[90]
HS11286 ΔMDR:ΔCPS	Strain HS11286 ΔMDR with an added deletion of the gene coding Capsular Poly Saccharide, rendering the strain less mucoid due to the loss of the capsule in order to enhance further genetic manipulation	YES	Complete genome sequencing done by Liu et. al. [105]
HS11286 ΔMDR:ΔCPS: Δ*mob1*	HS11286 ΔMDR:ΔCPS with the deletion of the *mob1* gene, a part of the T4SS-1 (VirB system) that is responsible for the	YES	Mutation constructed by Robeena Farzand, University of Leicester Lab212 in III Dept.

	mobilisation of DNA for conjugation by means of nicking dsDNA into ssDNA at the origin of transfer		
HS11286 ΔMDR:ΔCPS: ΔICE1	HS11286 ΔMDR:ΔCPS with the deletion of the entire ICEKp1 -62kb in size-(ICEKpnHS11286-1) which carries T4SS- (VirB system)	NO	Mutation constructed by Robeena Farzand, the University of Leicester Lab212 in III dept. ICE identified and characterised in work done by Zhang et al [144-146]
HS11286 ΔMDR:ΔCPS: Δ*mob2*	HS11286 ΔMDR:ΔCPS with the deletion of the *mob2* gene, a part of the T4SS-2 (tfc system) that is responsible for the mobilization of DNA for conjugation by means of nicking dsDNA into ssDNA at the origin of transfer	YES	Mutation constructed by Robeena Farzand, the University of Leicester Lab212 in III Dept.
HS11286 ΔMDR:ΔCPS: ΔICE2	HS11286 ΔMDR:ΔCPS with the deletion of the entire ICEKp2 -56kb in size-(ICEKpnHS11286-2) which carries T4SS- (tfc system)	YES	Mutation constructed by Robeena Farzand, University of Leicester Lab212 in III Dept. ICE identified and characterized in work done by Zhang et. al .[144-146]
ΔMDRΔKPCΔCPSΔVirB(1-2)ΔVirB(4-6)	HS11286 ΔMDR:ΔCPS with the deletion of the genes responsible for formation of the cylindrical part of the T4SS(VirB system) namely v*irB1, virB2 virB4 virB5* and *virB6*	NO	Gene deletion constructed by Yingzhou Xie, at University of Leicester Lab 212 , III Dept.
HS11286 ΔMDR:ΔCPS: ΔICE1:ΔICE2	Double mutant of both T4SS in HS11286 (VirB and tfc) or ICEKpnHS11286-1 and ICEKpnHS11286-2 respectively	NO	Mutation constructed by Robeena Farzand, University of Leicester Lab212 in III dept. ICE identified and characterized in work done by Zhang et. al. [144-146]

3.4.3.2 Secretion profile of the FIC domain bearing proteins:

The first assay was aimed to assess which of the five Fic proteins could be retrieved from the culture supernatant (secreted portion) once the temperature shift was done. Strain *K. pneumoniae* HS04160 and HS11286 were transformed with plasmid constructs carrying the genes corresponding to each Fic domain bearing protein under an IPTG-inducible promoter. The strains were incubated and treated as described in the materials and methods section. The secreted proteins were adsorbed into Strataclean resin after removal of bacteria and filtering the supernatant; this method was devised based on earlier research [39, 49, 55, 143, 147].

No secretion was observed for any of the Fic proteins except for the FIC-RL protein (negative data where western blot showed no FLAG tag Fic-RL protein in supernatant is not shown). Figure 3.4 (lanes 1 and 2) shows the experiment and a replicate where secreted proteins produced by *K. pneumoniae* transformed with the pWSK29-apra-vector carrying the *fic-rl* gene under an inducible promoter are analysed by western blot. Using monoclonal antibodies recognising the FLAG tag on these proteins, western blot analysis revealed a strong band of the expected size of FIC-RL in the secreted fraction. The same background bacteria (HS11286ΔMDR) that did not harbour this plasmid didn't show this protein in either compartment upon induction of secretion. Indicating that the secreted fraction in the supernatant was showing the expressed FLAG tag protein and this protein is not present (negative control) when this plasmid is not transformed into this strain.

After establishing that Fic-RL was secreted, the aim was to identify which secretion system is responsible for the secretion of this protein. Fortunately, parallel research in our group and collaborators has resulted in the production of various mutants lacking either both copies of the type 6 secretion system (T6SS), or lacking type 1 of Type 4 secretion system (T4SS-1) on integrative and conjugative element 1 (ICE1) and type 2 of the T4SS-2 present on ICE2 (table 3.2).

These mutant strains were transformed with the pWSK29-apra plasmid carrying the *fic-rl* gene and their ability to secrete the Fic-RL protein was assessed. Figure 3.4 also shows the secretion experiment done on the mutant that lacks two identified loci of the T6SS. No discernible difference could be detected between the two strains (WT vs T6SS mutant) as both seem to secrete this protein, which leads to the conclusion that type 6 secretion system is not responsible for the secretion of this protein.

Figure 3.5 shows assessment of 2 mutants each carrying a different type of T4SS in the strain HS11286. Strain HS11286 ΔMDR:ΔCPS: ΔICE1 has the deletion of ICE1 which carries the VirB type T4SS (see figure 3.7 for the map of each of these integrative conjugative elements), while strain HS11286 ΔMDR:ΔCPS:ΔICE2 carries the system dubbed as the tfc type (or type 2) T4SS. The T6SS mutant from the previous figure was included for comparison. The results show that the mutant lacking ICE1 cluster (carrying the VirB type T4SS) lost the ability to secrete the Fic-RL protein, while the wild-type background, T6SS mutant and the ICE2 mutants all were still able to secrete this protein.

Further confirmation that ICE1 T4SS was responsible for the secretion of FIC-RL was obtained when additional mutants of DNA mobilisation genes were tested. The genes (*mob1, mob2*) are relaxases that nick the dsDNA to produce ssDNA at the origin of transfer for DNA to be transferred through each secretion system. These two mutants have also been deleted by similar methods and were included in this assay (*mob1*, *mob2)* as described in table 3.2 as well as a double mutant that lacked both ICE1 and ICE2.

Results in figure 3.6 show that Fic-RL was not secreted by the ICE1 mutant or the double mutant of ICE1 and ICE2, while at the same time showed that the protein is present in the cellular fraction of these mutants (not shown), indicating that the protein is being expressed, suggesting that these two mutants (ICE1 and the double mutant) were no longer able to secrete this protein. Bacteria that secrete the Fic-RL protein did not show the protein in the intracellular fraction and only in the secreted fraction.

Further confirmation of this finding was obtained when a different mutant of the T4SS present on ICE1 was used (virB*1,2* and *virB4-6* genes were deleted in this strain), and results show that the two strains (ΔICE1 and Δ*virB1-2, 4-6*) both show no secretion of the Fic-RL protein.

It is noteworthy to mention that secretion experiments were accompanied by a measurement of autolysis done by quantifying free DNA as shown in the methods section using Nano drop technique of the supernatant and measuring the DNA level in the secreted fraction and comparing it to wild type secreted fraction without expression of Fic-RL at different time points during the time shift (1,2 3 and 4 hour points) . This was done to compare wild type (as non-transformed HS11286 with the any of the pWSK29 vectors carrying the Fic genes) with the mutant bacteria at the beginning of each experiment and to assess if any protein that was retrieved from the supernatant was the result of cell death and not actual secretion before continuing with the experimentation.

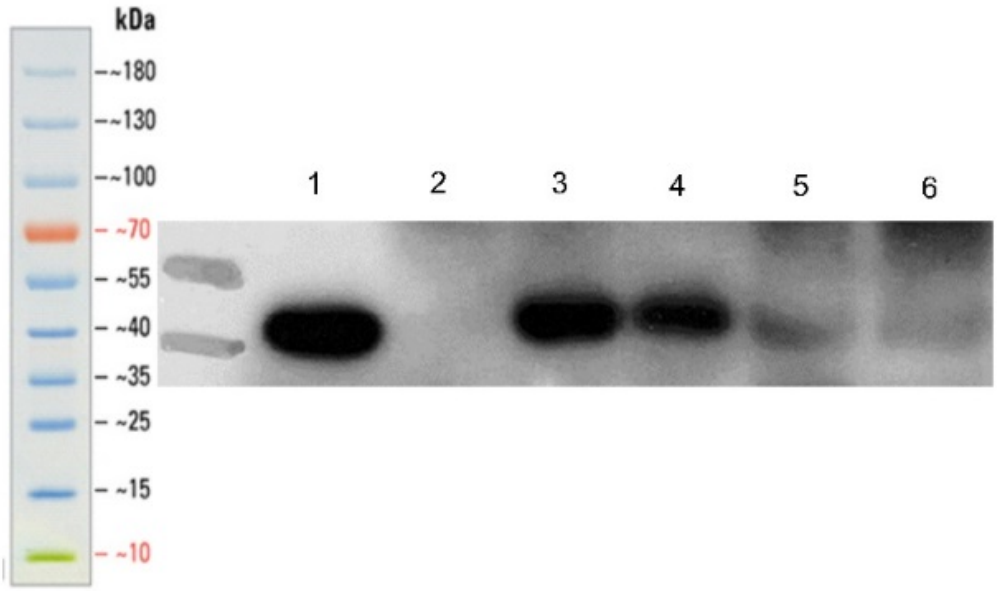


Figure 3-4 Analysis of proteins produced and secreted by different *Klebsiella* strains using western blot analysis with anti-FLAG tag antibodies. Lane 1 shows secreted part from strain HS11286 ΔMDR (representing the wild type) transformed with pWSK29-Apra-*fic-rl.* Proteins were isolated from cultures supernatant following induction of its secretion, lane 2 represent the negative control where the same method have been done to the bacteria that had not been transformed with the plasmid. Lane 3 is a repeated experiment on the wild type bacteria with the plasmid showing the secreted proteins, whereas lane 4 shows the secreted proteins from of HS11286 mutant lacking the T6SS locus1 and locus 3 double mutants (complete knockout by deletion of the T6SS machinery), lane 5 and 6 show the corresponding samples in lane 3 and 4 respectively, however, the protein sample analyzed here is that of the intracellular proteins obtained from sonicating the pellet. Bands shows in this film are all the expected sizes of the FIC-RL protein, as predicted. This indicates that the Fic-RL protein is in fact a secreted protein evident by the fact that the protein is retrieved abundantly from the secreted fraction, furthermore this experiment indicates that the deletion of T6SS does not interfere with the secretion of Fic-RL protein in *K. pneumoniae*.

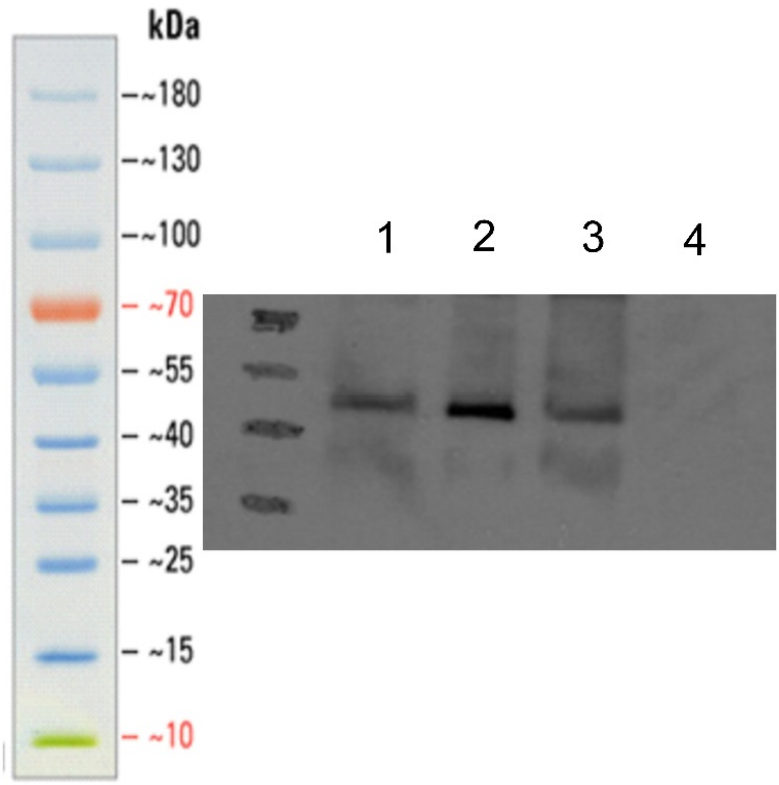


Figure 3-5 analysis of proteins secreted by different *Klebsiella* strains transformed with the pWSk29 plasmid carrying the *fic-rl* gene under an IPTG-inducible promoter (a) proteins carrying a FLAG tag on the C-terminal end was detected using anti-FLAG tag monoclonal antibodies. Here a western blot was done in a similar manner to that shown in figure 3.4, in lane 1 the tagged FIC-RL protein in the secreted fraction in the wild type bacteria (HS11286 ΔMDR:ΔCPS), lane 2 similarly done but in the T6SS mutant (HS11286 ΔMDR: ΔT6SS-1: ΔT6SS-3), lane 3 shows proteins secreted by T4SS mutant on ICE2 (HS11286 ΔMDR:ΔCPS:ΔICE2), while lane 4 shows in the T4SS mutant present on ICE1 (HS11286 ΔMDR:ΔCPS:ΔICE1).

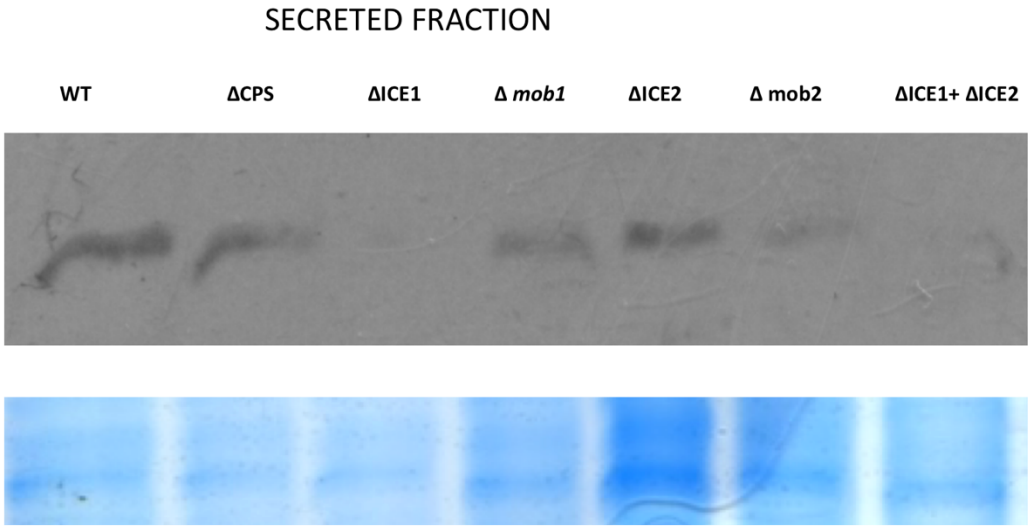


Figure 3-6 Secretion analysis incorporating various mutant strains of various deletions of the T4SS. Similar to the secretion assay done in figures 3.4 and 3.5, in this experiment the mutants used were expanded to include deletion mutants of ICE1, *mob1* (DNA mobilization gene present on ICE1), ICE2, *mob2* (DNA mobilization gene present on ICE2), a double mutant lacking both ICE1 and ICE2, and lastly a mutant lacking only the genes coding for the cylindrical part of the T4SS present on ICE1 (VirB type secretion system). Below the secreted fraction, image is the corresponding SDS-PAGE gel of secreted fraction. The Western blot reveals that FIC-RL was not secreted by any strain that lacks ICE1 (ICE1 mutant or the double mutant), in addition the mutation of *mob1* gene present on ICE1 (or the homolog gene on ICE2) does not affect the secretion of Fic-RL, both these genes code for proteins that are responsible for DNA mobilization and not protein secretion. Looking at the intracellular fraction, it was revealed that the Fic-RL it was in fact "trapped" within the cell unable to secrete it (not shown). Further confirmation was shown by negative secretion of Fic-RL from a mutant that has genes responsible for the cylindrical part of the T4SS present on ICE1 (and not the entire cluster) deleted (not shown). Further confirming the that T4SS machinery present on ICE1 is responsible for the secretion of Fic-RL

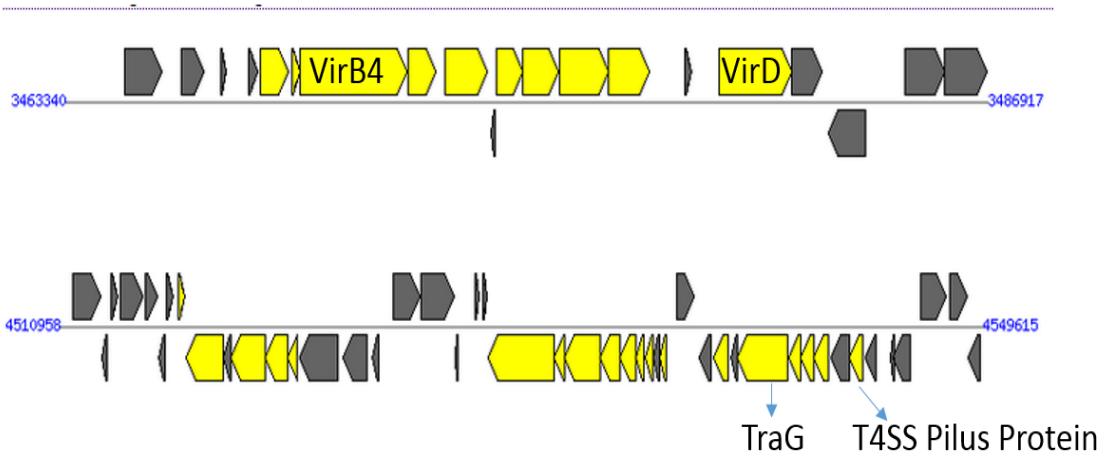


Figure 3-7 Gene clusters in *K. pneumoniae* coding for T4SS. Many subclasses of the T4SS are present on gene clusters, here in strain HS11286 two gene clusters are revealed that carry genes encoding two different subclasses of T4SS machinery, the first (top) is a VirB4/VirD system present on ICEKp1 and the second (bottom) is tfc operon carrying a T4SS (tra) subclass on ICEKp2 element. These two elements have been identified using online databases that track T4SS (Secret4[134]) and ICE (iceberg[135]). Image retrieved from the Secret4 website

3.5 Discussion

Bioinformatics analysis of genes coding for Fic domain bearing proteins in *K. pneumoniae* reveals the diversity of these genes. First Fic proteins can be coded by genes that seem to be only carried on pathogenicity or genomic islands (*fic-gn*) or elements that have very low penetrance in this bacteria's population that seem to be inserted in transposon-like fashion in the genome (such as *fic-re*). Both two genes (*fic-gn* and *fic-re*) are poorly distributed among *K. pneumoniae* as shown in the sequence data of nearly 60 strains on the NCBI database, which further hints to them being acquired genes.

The toxin (*doc*) and its anti-toxin (*phd*) (TA) encoding genes were shown to have a nearly 90% prevalence which comes as no surprise (as shown in figure 3.1 A). Two major theories explain the high prevalence of TA systems that are chromosomally encoded, both of which support the high prevalence of these genes. The first theory postulates that these TA systems are coded in key or vital regions of the genome, where deletions in this area would be catastrophic (more so due to the loss of the anti-toxin within this region) [148-150]. Further inspection of the region in *K. pneumoniae* where the TA system is present does indeed reveal important cellular function genes located proximal to *doc/phd* (coding for ABC transport system and sugar metabolism genes).

The other major theory proposes that the chromosomally encoded TA systems serve as anti-addiction modules, where if the bacterial cell takes up an addiction type plasmid containing a homologous TA system (Doc/PHD), the loss of the plasmid would not lead to the addiction effect by which the bacterial cells that lose the plasmid are killed[151]. A support for this theory in this particular regard (the Doc/PHD system derived from P1 bacteriophages) is classically described as an addiction system causing cellular arrest if not rescued by the anti-toxin PHD [152]. This particular TA system has been shown to enhance bacteria's virulence helping the bacteria enter a dormant state in response to overwhelming outside stress[153] maintaining its persistence and consequently its

pathogenicity. The new data supporting this data is shown by Doc toxin's function on the elongation factor T (EF-Tu) [38, 46] resulting in arrest in cellular function.

Further information about the *fic-ad* gene status makes more analysis increasingly difficult, as this gene is present in over half of the population of *K. pneumoniae*. There are very few hints to reveal the status of this gene given from the gene context, apart from being downstream from a 16sRNA gene (commonly used for species identification using sequencing) and a gene coding for Penicillin Binding Protein (PBP).

The *fic-rl* gene shown to be a conserved gene among all sequenced strains (also shown to be present in a small collection of 23 clinical isolates of *K. pneumonia* using the detection primers). Although the definition and significance of conserved genes in bacteria is a topic of debate, it remains clear that they are DNA sequences that through evolution remained unchanged either because the loss or change of these DNA sequences has lethal consequences on the fitness, survivability or –in this case- the virulence of the bacteria, which would be significantly less that might cause an evolutionary negative impact. These sequences thus are of high value that ranges from essential genes to highly conserved sequences that are of high value to the organism [154, 155]. Having *fic-rl* in 100% of isolates tested (and present on databases) points to this gene being a significant player in this bacterium's functions. The secretion of the protein coded by *fic-rl* gene combined with the high conservation of this gene hints at the evolutionary value of this effector in the virulence of *K. pneumoniae* (as would be further elucidated in subsequent chapters).

Assessing Fic-Rl's role was aided by a literature search of published data of other Fic proteins in other prokaryotes. Other bacteria have been shown to use Fic proteins as virulence factors that are often secreted into the host cell to modulate effects that help to maintain the bacteria's infection of the host cell. An example of this is the T4SS effector of *Legionella* (AnkX), which once secreted into the host cell, causes a vesicular diversion that ultimately leads to maintenance of intracellular infection by the bacteria [40, 45, 48]. Other secreted effectors, however, cause cytoskeletal collapse and direct

cytotoxicity (such as caused by IbpA and VopS)[43, 47, 60], consequences of the direct cytotoxicity can possibly lead to evasion of host defences[56]
Initial results showed that out of the five Fic proteins present in *Klebsiella,* only Fic-RL was secreted. This leads to two possible conclusions, first is that secretion assays shown in the literature that relies on temperature shifts are usually successful in showing secretion of toxin proteins (usually that target is a eukaryotic host). Secondly showing no secretion (of the four other Fic proteins) using this method does not exclude they are not secretion system effectors (or secreted proteins by other means), it only indicates that that temperature is not a necessary signal for their secretion or delivery into the target host.

Doc toxin was shown by this experiment to not be a secreted protein (using our method. However, Doc toxin is generally believed to not be a secreted protein, rather it is expressed within the target host by means of plasmids carrying the coding sequence for this gene that once delivered into the target cell the expression of the Doc toxin is driven (with resulting death for the target cell if it does not contain the anti-toxin PHD) [37, 156, 157].

Fic-RL secretion was not affected in a mutant that lacked both loci coding for the T6SS in strain HS11286. This finding in retrospect was not surprising, as usually effectors for this secretion system are involved in interbacterial competition and antagonism[80, 103] by targeting bacterial cell targets (Peptidoglycan layer, cell membrane or DNA assembly mechanisms[158-160]) and very rarely is a mechanism for secreting virulence toxins targeting eukaryotic cells[161]. Other discovered functions of T6SS effectors include cell to cell signalling and biofilm remodelling, both of which are also mechanisms not directly responsible for virulence in which Fic proteins are involved in[162, 163].

Secretion of Fic-RL was not affected in mutants of (T6SS, T4SS Tra system on *icekp2*), except that on T4SS on ICE1 (*icekp1)* seen in the ΔICE1 mutant, mutant of ΔICE1 and ΔICE2 and later in a mutant of a small segment of ICE1 coding for core genes of the T4SS present on that island were all unable to secrete Fic-RL. These results indicate that the T4SS present on ICE1 is responsible for the secretion of the Fic-RL protein.

This finding provides insight on the function of the T4SS (VirB4/VirD system), where secretion systems are shown to either secrete DNA or proteins or both [91], however, it is suggested in the literature that *icekp1* (ICE1) is responsible for DNA conjugation as shown by experimental work revealing the ability of ICE1 machinery to successfully integrate and conjugate the coding DNA sequence to another bacterial host [164]. Considering these findings, it indicates that the T4SS present on ICE1 secretes both DNA and proteins. This is shown by parallel research which showed that the ICE1 mutant bacteria lose their ability to transfer DNA by conjugation which was measured to be a significant loss (see table 4.3). As the previous classification of these systems was done according to the type of effector to characterise their function (DNA uptake, DNA secretion and virulence protein secretion[91]).

The VirB4/VirD T4SS system have been shown to be an essential factor in pathogenicity in bacteria that carry these system[165] and even conjugation of DNA into eukaryotic cells ([147, 166] as well as pure conjugation of DNA among species[167-169] and is shown to carry out both functions[169]. The VirB4/VirD type T4SS of *Klebsiella* secreting the Fic-RL protein indeed goes in tandem with other information regarding this secretion system especially its role as a pathogenicity factor. Further studies of the pathogenicity of Fic-RL protein as an effector of this secretion system will be discussed in subsequent chapters.

Chapter 4

Virulence Analysis

4.1 Introduction:

As was discussed in the previous chapter, Fic proteins are often delivered into the host cell by means of specialized secretion systems (often T4SS or T3SS). Once these Fic domain bearing effectors are delivered into the target cells, they modulate effects that would promote the virulence of the invader, via several possible mechanisms.

The mechanisms by which the Fic proteins enhance the virulence of the bacteria can be revealed by examining the targets of which these proteins affect. So far, a few targets have been identified. The first described target of two Fic proteins; VopS (*Vibrio parahaemolyticus)* and IbpA (*Histophilus somni*) proteins, is the small Rho GTPases. The effects of the delivery of VopS and IbpA into the host cells can be detected by viewing the destabilization and collapse of the actin cytoskeleton and resultant cell death.

For IbpA, *H. somni* strains that expressed this protein caused the cytoskeletal collapse and cytotoxicity in Bovine Alveolar Type 2 cells (BAT2) by targeting Rho GTPases, however bacterial strains that lacked this protein did not cause apparent cytotoxicity in BAT2 cells[53]. Strains that lacked IbpA where unable to migrate between layers of BAT2 cells unless the cells where pretreated with IbpA. Moreover, antibodies produced against IbpA protected the BAT2 monolayers from bacterial migration [51, 53, 60]. Earlier studies showed that antibodies to IbpA protected cattle from pneumonia caused by *H. somni* [170], and this is explained by the subsequent research that revealed the pathogenic molecular mechanism of this protein.

In the case of VopS, from *V. parahaemolyticus,* the story is very similar. The protein is delivered by a T3SS (T5SS in the case of IbpA which utilizes the Sec system to cross proteins through the inner membrane), and once inside the cell, it causes cytotoxicity manifested as actin cytoskeleton collapse. The cytoskeletal collapse was shown (as is the case of IbpA) to be caused by the AMPylation of the Rho GTPases by VopS resulting in their inability, following AMPylation, to bind to their downstream effectors (such as PAK) [43, 47].

Other G proteins, such as the Rab GTPases, are also targets of Fic proteins; however, the effect of targeting these proteins does not seem to result in direct cellular cytotoxicity as is the case for Fic proteins that target Rho GTPases. AnkX protein of *Legionella* is the example described so far utilizing this mechanism. Once introduced by means of T4SS, AnkX performs phosphocholination on its target Rab GTPases. AnkX protein helps the pathogen to establish infection vacuoles by disrupting the actin dependent vesicular trafficking system (controlled by the Rab GTPases). This prevents the destruction of internalized bacteria, and establishes immature vacuoles containing bacteria, and ultimately helps the pathogen to evade the intracellular defenses [40, 45, 55].

Doc-Phd toxin-antitoxin system (Doc toxin is the Fic protein in the pair) have been proposed to not act directly as virulence factors but helps the pathogen to escape hosts defenses and antimicrobial agents indirectly by inducing bacteria to fall into a dormancy state. In the presence of the anti-toxin the bacteria grow. However, some environmental factors that would cause the degradation of the anti-toxin (such as presence of antibiotics) ultimately lead to the presence of more toxin than antitoxin. The presence of more of the Fic toxin protein Doc, would cause the bacteria to enter a more resistant form[171].

4.2 Aims

To assess the contribution of the five Fic homologs in *K. pneumoniae* to its virulence.

4.3 Results

4.3.1 Mutant Construction:

Following the identification of Fic protein homologs in *Klebsiella pneumoniae*, a strain that houses several genes coding for Fic proteins was chosen for further experiments. Each of the genes encoding for the Fic proteins was knocked out or deleted. The resulting strains were assessed in a variety of assays to elucidate the functions of these proteins.

Sequence analysis revealed that strain HS04160 housed one of the most prevalent Fic domain proteins (*docKp* an orthologue to Doc toxin-antitoxin systems present in other bacteria), and it was the only strain to house *fic-gn* within the context of a genomic island, in addition to *fic-rl* the conserved gene coding for the Fic-RL Fic protein. Three different single mutants were constructed in this strain of each of the three homologs (Δ*fic-rl,* Δ*fic-gn* and Δ*dockp*), as well as a fourth mutant deleting the entire KpGI-2 Island that housed the *fic-gn* homolog. The deletion of the entire KpGI-2 Island was done in order to verify any phenotype that results from deleting the *fic-gn* gene (genomic islands usually carry genes that have similar functions such as fitness genes or pathogenicity genes or drug resistance genes)[172, 173]. A second strain HS11286 was also used to construct a mutant of the *fic-rl* gene (Δ*fic-rl*). This strain had been used in a parallel study that focused on the Integrative and Conjugative Elements (ICE) and their relationship to Type IV Secretion Systems (as discussed in the previous chapter). Within this study, mutants were constructed to delete ICE1 that housed T4SS genes, and ICE2 that also housed different homologs and complimentary genes of T4SS present in ICE1, as well as a double mutant deleting both ICE1 and ICE2 and mutant that deleted the genes that encode the cylindrical proteins of the T4SS (VirB4 and VirB6). The deletion of

fic-rl in HS11286 allowed us to hone on in which secretion system was responsible for secretion of the Fic-RL protein.

The conjugation based allelic exchange method was used to delete the KpGI-2 island (described in [106]). This method is superior to the lambda red method for larger deletions (KpGI-2 is almost 6.3 kb in size) Figure 4.1.

The Lambda red method, which is also described in length in the materials and methods section, was used to produce the remaining mutants used in this study. An SOE PCR produced DNA fragment (similar to what was produced in figure 4.1) was introduced into the bacterial strain that hosted the gene to be deleted. The same strain would be prepared to host a plasmid coding for the bacteriophage λ red genes (*exo, bet* and *gam*). These genes were induced to produce the proteins, and cells that contain these proteins are prepared for competence to receive the produced SOE-PCR DNA fragment. These λ red proteins allow recombination to take place. The resultant mutants were screened by PCR using genomic DNA as a template and external primers (primers that lay outside of either flanking region). Following the capture of the correct mutant, the antibiotic cassette was removed to obtain a marker-less mutant by means of flippase recombination (also discussed in the methods section). All mutants were constructed to be inframe deletions by removing the coding sequence from start to stop codons. Figure figures 4.2 and 4.3 shows the final electrophoresis gel analysis of PCR-amplified DNA fragments from for these mutants. Mutants were also verified by sequencing.

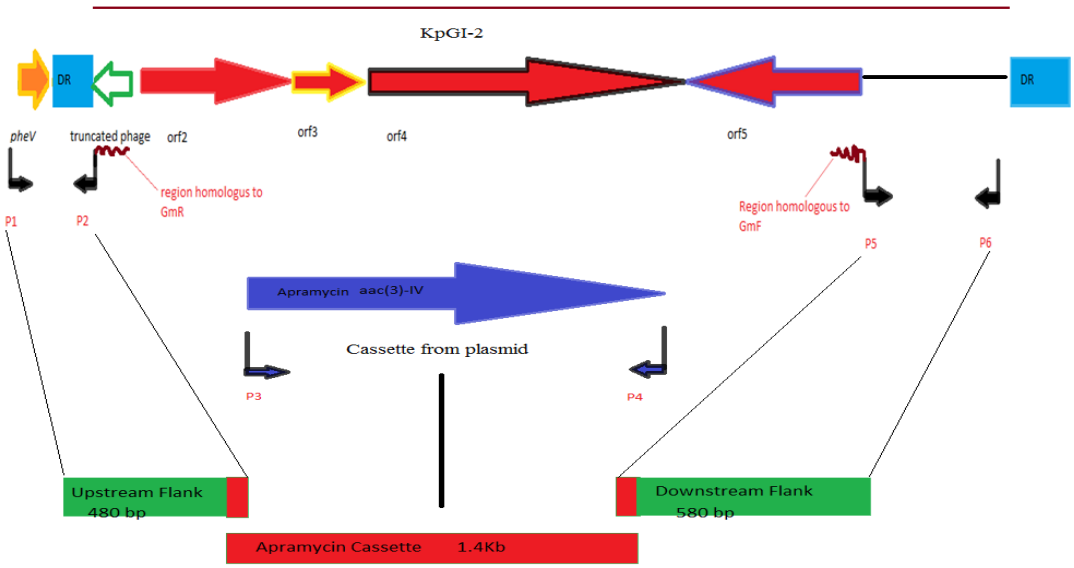


Figure 4-1 Allelic exchange strategy used to delete KpGI-2.

On the top part, a genetic map of KpGI-2 is represented with arrows indicating the 5 Open reading Frames of the Island (orf5 is fic-gn); direct repeat sequences (DR) are shown in blue boxes; the left DR is preceded by the pheV gene (a common insertion point for Genomic Islands) which is followed by the 5 ORFs of KpGI-2. Primers P1 and P2 were used to amplify the left flanking region of KpGI-2Orf 1-5, while primers P5 and P6 were used to amplify a right flanking region. Primers P3 and P4 (AKA GmF and GmR) were used to amplify the gene of the antibiotic cassette from the plasmid pJTOOL-8 (coding for Apramycin resistance). A homologous sequence was attached to P2 and P5 to that of GmR and GmF respectively (the red part introduced into each flank) this will introduce complimentary regions in each flank to direct them to ligate during SOE PCR. Primer sequences are shown in the materials section. The resulting SOE PCR fragment was used to construct a suicide vector, (which was introduced by means of conjugation) into the *K. pneumoniae* strain HS04160. Single cross over event is selected by means of selection with apramycin (coded on the antibiotic cassette) and the Double cross over event was later selected by means of counter selection with *sacB* gene, present on the backbone of the suicide plasmid. In the presence of sucrose *sacB* gene, which produce levansucrase enzyme, causes the death of the cells due to a toxic by product. Cells that underwent double cross over would be apramycin resistant and able to grow on sucrose containing plates.

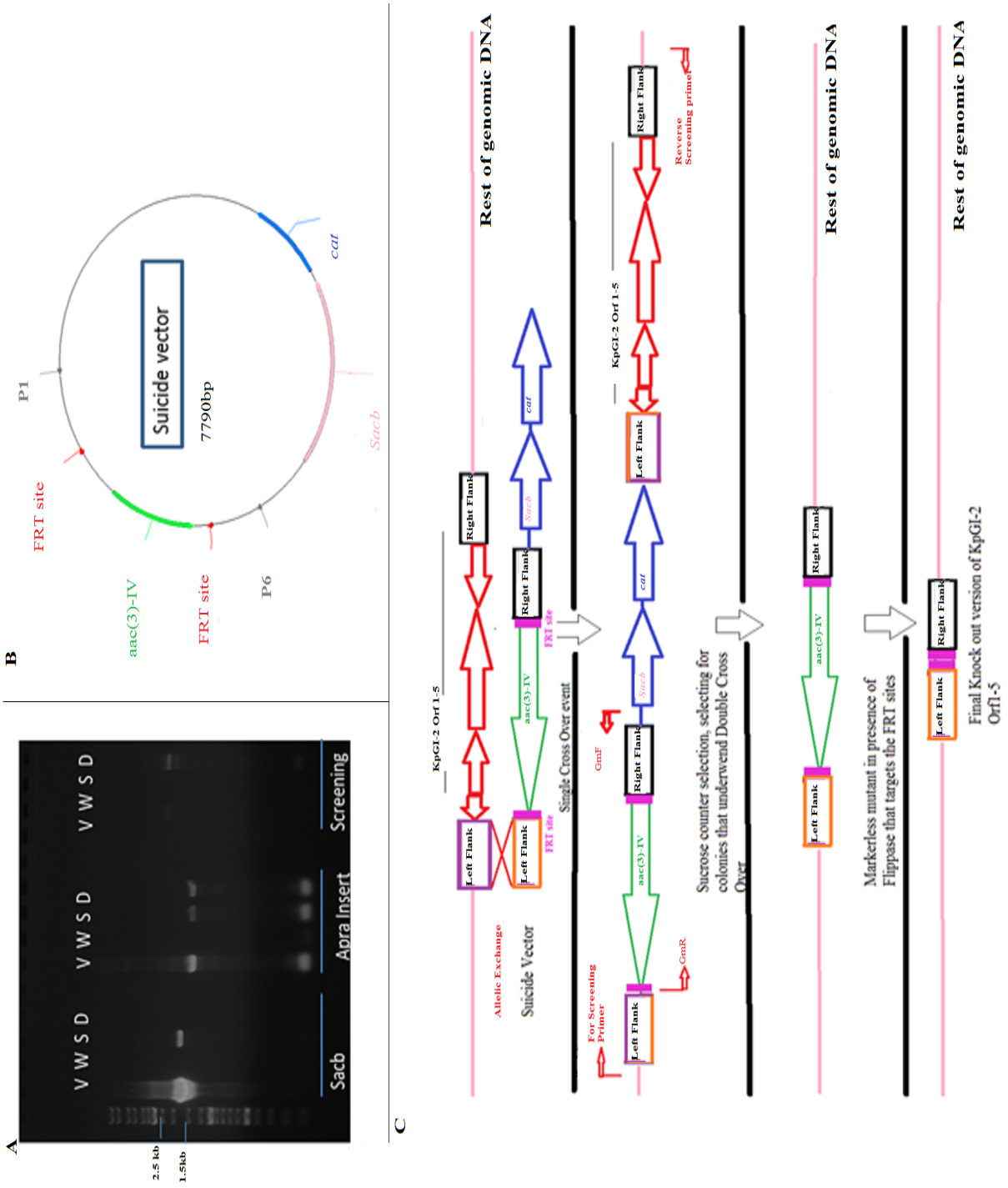


Figure 4-2 Knockout mutant deletion by inframe insertion deletion using allelic exchange method. in 2.2, A. Gel electrophoresis of PCR-amplified DNA fragments from each of the stages of the mutatgensis. The progress of the mutation goes from the suicide vector [V], its introduction into the Wild Type strain [W], selecting for a Single Cross Over mutant [S] followed by sucrose counter selection for a Double Cross Over mutant [D] with the final replacement of KpGI-2 Orf1-5 with the apramycin resistance cassette (*aac(3)IV*) labelled as Apra insert. First four lanes show the *sacB*

gene used for counter selection, which is carried on the suicide vector backbone and is expectedly present on the vector and the single cross over mutant and not in the untransformed wild type strain or the double cross over mutant. The last four lanes show the screening primers that lay outside of the target regions' flanks (Left and Right flanks) and serve to screen the target area for mutation (KpGI-2-Screen-F and kpGI-2-screen-R shown in table 2-3). It is negative (no band) in the wild type (too large to amplify with Gotaq, however, it was amplified using KOD-not shown-), it is also negative in the single cross over mutant (too large, more than 15kb in size). The Final double cross over mutant shows the correct sized band expected of the region following Knockout of KpGI-2 Orf1-5 (2.5kb). (4.2.B) shows the general structure of the suicide vector. Color coded genes shown are the *aac(3)IV* apramycin resistance cassete (green), s*acB* (pink),and *cat* the cholramphenicol resitance gene (blue). In (4.2.C) a general schematic of the conjugation based allelic exchange method is shown. When the suicide vector is introduced into the wildtype strain via conjugation, the allelic exchange occurs at either of the homologous flanking regions (Left flank and Right flank). In this schematic, the left flank was chosen. A single cross over mutant results with recombination occuring at the left flank (although two types of integration can occur Left sides or Right sided orientation) the left sided integration is shown for simplicity. The entire suicide vector is incorporated into the genome following recombination at the homologous flank (the single cross over mutant will contain both s*acB* and *aac(3)-IV*). During sucrose counter selection s*acB* in the presence of sucrose produces a toxic by product that eventually eliminate cells with this type of mutation. In cells that underwent a double cross over (a second recombination event at either of the two homologous flanks) will result in the removal of the plasmid backbone along with the target region (KpGI-2 Orf1-5). Such mutant is selected with apramycin plates, but it is sensitive to chloramphenicol. A markerless mutant (removal of the *aac(3)-IV* marker) was constructed by introducing pFLP-2; a plasmid containing the Flippase gene that targets the FRT sites and removes the region bracketed by these sites. This was confimed later by sequencing using the screening primers.

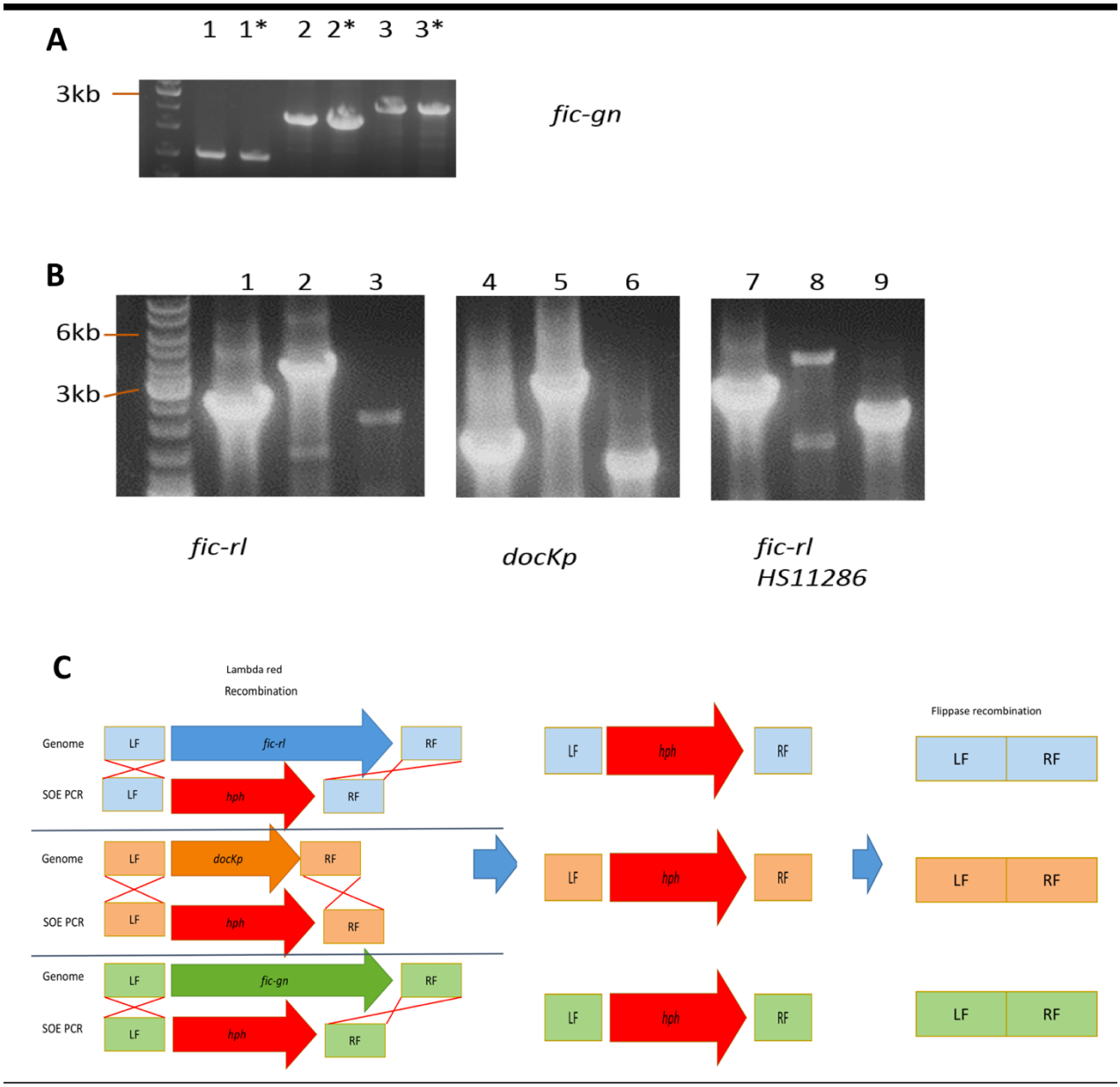


Figure 4-3 Mutant construction by inframe insertion deletion using Lambda red recombination. 4-3.A shows the electrophoresis analysis of PCR-amplified DNA fragments showing bands confirming the deletion of the *fic-gn* homolog in HS04160 (*orf5* of KpGI-2). This particular experiment tested two colonies simultaneously and thus shows a replicate for each band (marked with *). The image shows from left to right the deletion of *fic-gn* gene in HS04160. The last two lanes on the right (3 and 3*) show the wild type strain genomic DNA amplified with the screening primers that lay outside the flanking region selected for the mutation (on two different colonies that were tested), whereas the lanes in the middle (2 and 2*) show the mutants that carry

the DNA sequence of the antibiotic marker gene in the same region, the DNA fragment for the antibiotic marker gene is slightly smaller than the original gene (the green arrows in the bottom image indicate the region deleted) and shows the correct size bands. In the lanes on the far left (1 and 1*) show the bands corresponding to the DNA fragments that were amplified using the same primers in the marker-less mutant following flippase mediated recombination to remove the antibiotic marker. Figure 4-3.B shows from left to right the deletion of *fic-rl* gene, and *docKp* gene in strain HS04160 and the *fic-rl* mutant in strain HS11286. The first lane on the left in each of those images (1, 4 and 7) shows the wild type strain genomic DNA fragments amplified with the screening primers that lay outside the flanking region selected for the mutation, whereas the lanes in the middle (2, 5 and 8) show the mutants that carry the DNA sequence of the antibiotic marker in the same region. In both cases the gene for the antibiotic marker gene is larger than the original gene and shows the correct size bands. In the lanes on the far right (3, 6 and 9) the bands that amplify using the same primers in the marker-less mutant following flippase mediated recombination to remove the marker. 4-3.C shows a colored arrow scheme which depicts the mutagenesis process in lambda red recombination for those genes (approximate sizes are shown by arrows, flanks for each gene showed by a different shade of the same colour of the arrow depicting the gene). SOE-PCR of the two flanks was made with the antibiotic cassette intervening between the two flanks. When introduced into the induced and competent cells, a recombination event ensues at both of the flanks replacing the intervening part in the wild-type (the gene that is to be deleted) with the antibiotic cassette of the SOE PCR product. Lastly flippase mediated recombination removes the antibiotic marker gene leaving (by recognizing flippase Recognition Target sites at each end of the antibiotic cassette that were introduced by PCR) the two flanks with an inframe deletion of the original gene.

4.3.2 Bacterial viability and growth assessment:

Determining the rate of bacterial cell growth and viability of bacteria was the first assessment done on the mutants constructed. This was aimed to detect any negative

effects on the bacterial growth that a gene deletion in each of these mutants may have caused, as compared to their wild-type background strain. Such assays can provide insight to a possible phenotype that affected the fitness, survivability or the ability to thrive in rich media. They can also give a hint of any polar effects that may have resulted from unintentional frame shifts or promoter/induced inhibition of certain viability genes during mutagenesis that would otherwise mask any observable effects, or in the case that any of the deleted genes were contributing to the fitness of these strains and would produce false negative results when tested in virulence assays.

Figure 4.4 shows the results of these experiments performed in triplicates, where the wild type bacteria and the mutants constructed in the previous section were grown in LB broth and their growth was assessed by using the absorbance based method described in the methods section. Bacteria were inoculated to an equal starting absorbance measure of 0.2 and then optical densities of the cultures were recorded overnight using a Varioskan plate reader under the criteria and recording programmed into the software. CFU counting at different specific time points was also done. The results showed that the mutations had no effect on the bacterial growth or viability, as shown by CFU counting alongside the growth curves. This result is in agreement with what is described in literature, where FIC-domain bearing proteins were not noted to be affecting bacterial growth or survival, but serve as virulence factors[174]. These measurements were done for the wild type HS04160 and the 4 mutants constructed in this strain.

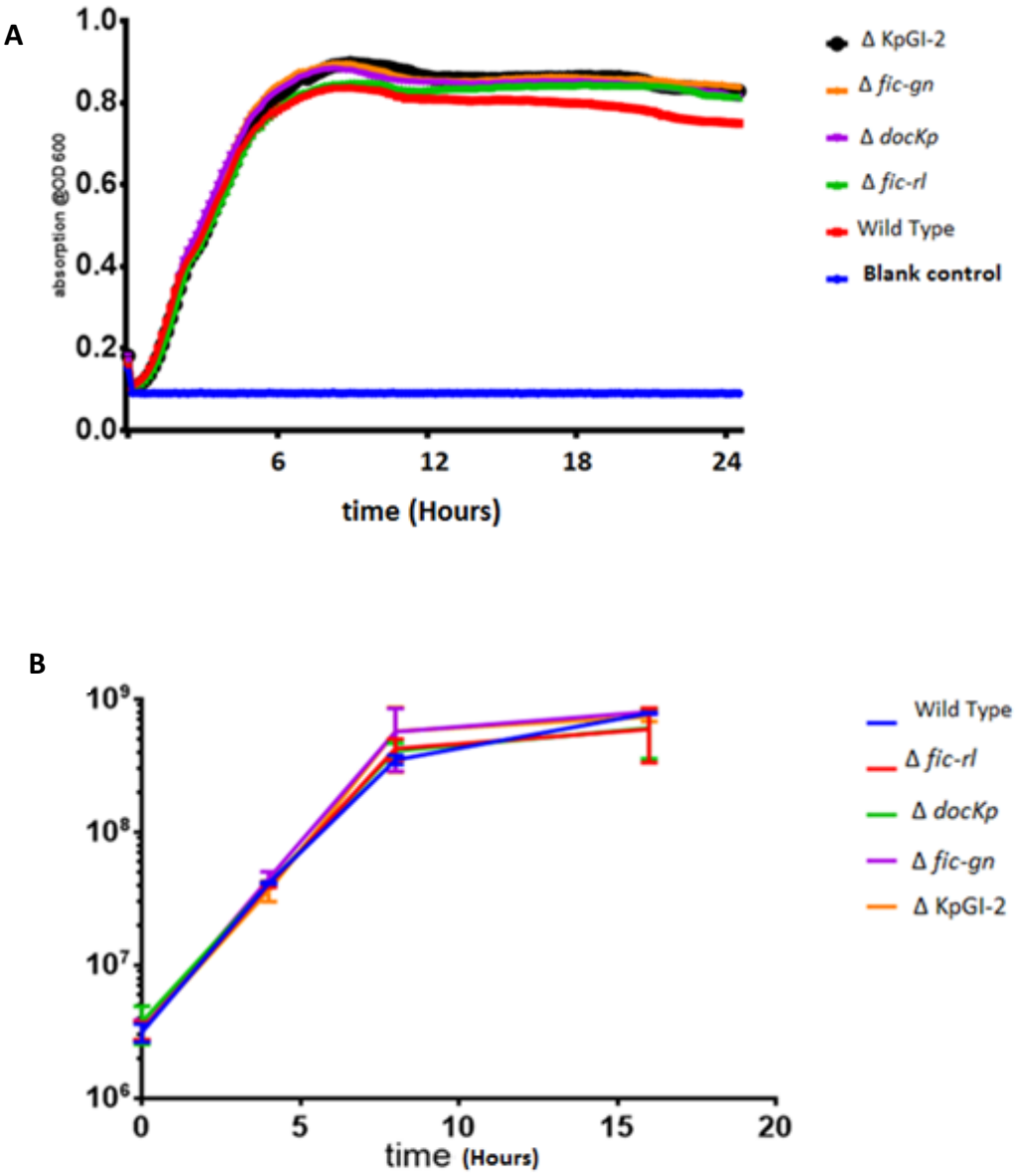


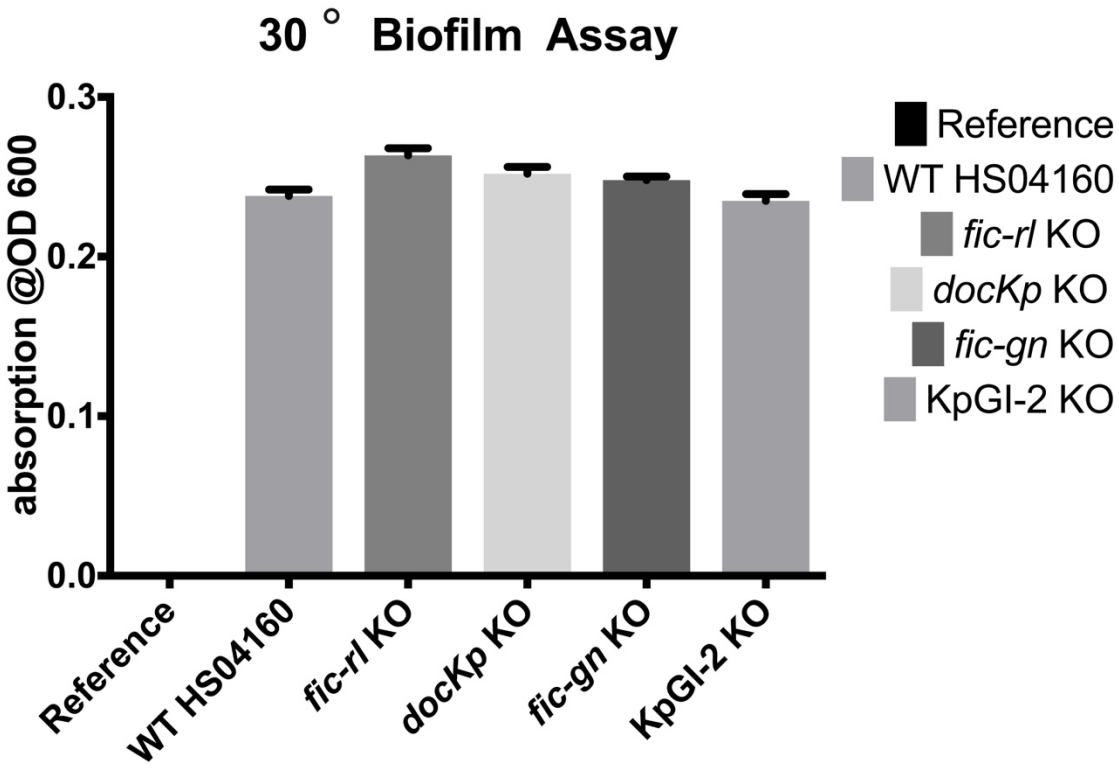
Figure 4-4 growth curves and viability assays. (A) Growth curve in LB plot from obtained absorbance readings over a 24-hour period with a reading taken every 10 minutes using a Varioskan plate reader (including error bars for each point). The four single mutants of the FIC genes and the wild type background HS04160 were tested multiple times. No significant growth impact for any of the mutants as compared to the wild type was observed. Thickness of each curve is due to error bar reporting on each reading from each replicate test. These results were consistent with the bacterial viability data obtained from the same cultures for an overnight growth period as measured by CFU counting over multiple dilutions shown in (B).

4.3.3 Biofilm formation

Biofilm formation is thought of as one of the common methods for bacteria to weather and endure the hosts' defenses; hence increasing the virulence of the species. Assessment of the biofilm formation can be regarded as a first-hand requirement to assessing bacterial virulence. To study whether the *fic*-genes have a role in biofilm formation, especially given that virulence attenuation is observed on the killing assay (shown in the subsequent sections), a simple assay was used to assess the formation of biofilm. This was achieved by growing the bacteria in 96 well plates in a stationary culture, at different temperatures over a set period of time. This allows the bacteria to "settle in" and attach themselves to the surface by forming the extracellular matrix that is the biofilm on the plate. The assay was performed under two temperatures conditions representing host body temperature 37^{o} C, and 30^{o} C. Biofilm formation by different *Klebsiella* strain was assessed by staining, as described in the methods section (figure 4.5).

The biofilm assay revealed little or no significant difference on the formation of the biofilm by any of the mutants as compared to the wild type strain. This suggested that the FIC-domain bearing proteins' function within the target host cell has no role in survival through formation of biofilm (figure 4.5).

A


30 ° Biofilm Assay
absorption @OD 600
0.3
0.2
0.1
0.0
Reference
WT HS04160
fic-rl KO
docKp KO
fic-gn KO
KpGI-2 KO
Reference
WT HS04160
fic-rl KO
docKp KO
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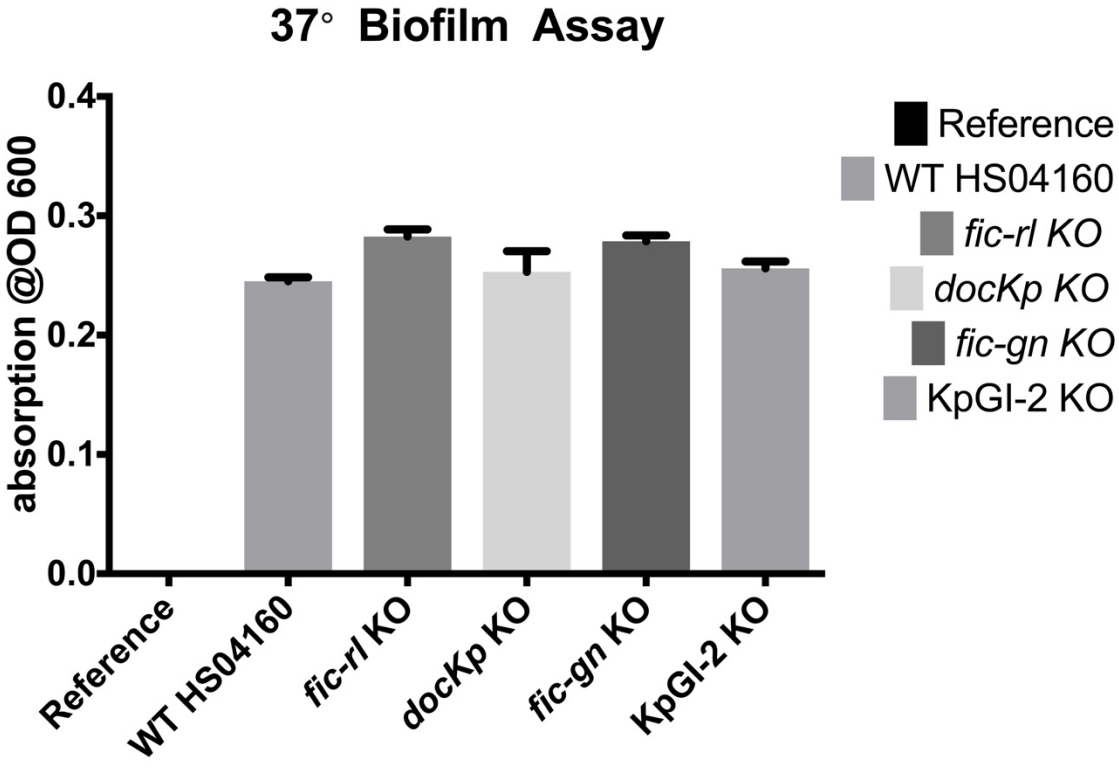
B


37° Biofilm Assay
absorption @OD 600
0.4
0.3
0.2
0.1
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Reference
WT HS04160
fic-rl KO
docKp KO
fic-gn KO
KpGI-2 KO
Reference
WT HS04160
fic-rl KO
docKp KO
fic-gn KO
KpGI-2 KO

Figure 4-5 Biofilm formation assay by different *K. pneumoniae* strains. Standard or blank used was fresh sterile LB broth. The assay was done at two different temperatures (30 ^{o} or 37 ^{o}) to assess if mutations of the different Fic genes had any effect on the formation of biofilm. Each experiment included cultures in 8 different wells in a 96 well plate and done in 3 replicates. Bacteria were cultured overnight (16 hours) in the wells and the formation of the biofilm is assessed the next day as mentioned in methods sections after being left stationary in the appropriate temperature incubator. The wild type *K. pneumoniae* HS04160 and the four single mutants were repeatedly tested in 3 replicates each. The average readings-means-were plotted after obtaining all the data; error bars indicate standard error of the mean (SEM). The experiment shows that these mutation does not reduce the bacteria's ability to form biofilm, nor is there a large observable difference in the formation of biofilm.

4.3.4 *Galleria mellonella* killing assay.

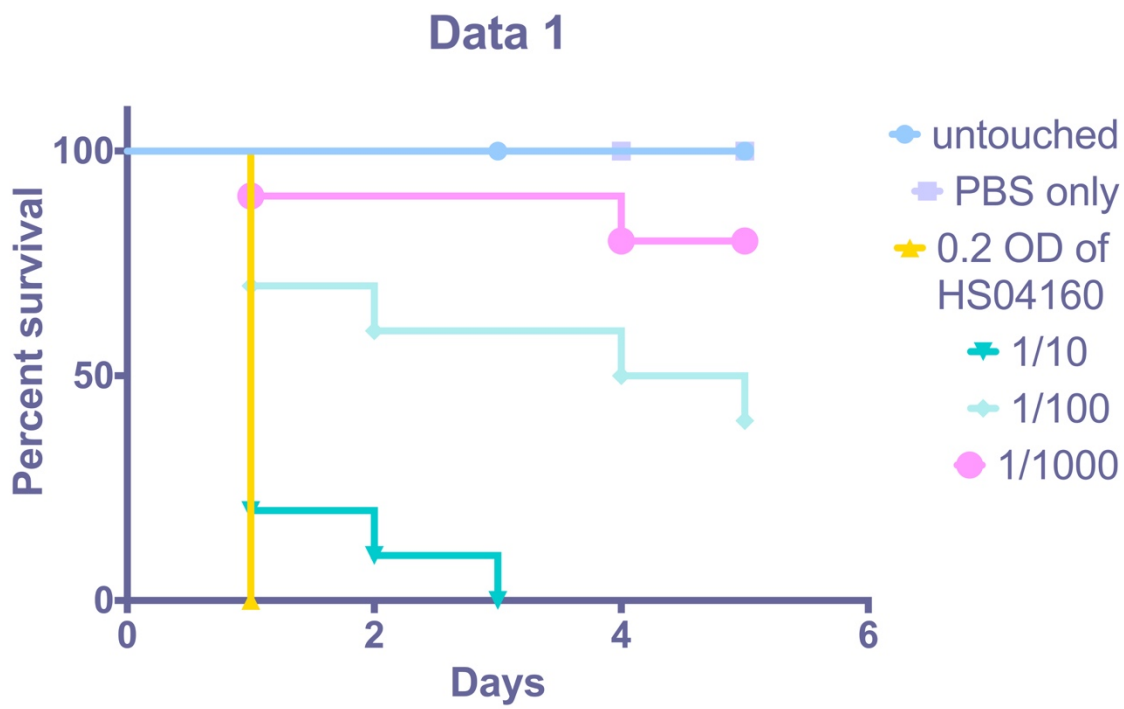
Any model assay for the study of virulence of bacteria and the effects of mutations involve simulation of an infection and the assessment of the ability of the bacteria to kill the host at different conditions or concentration. Commonly used assays have employed mammalian models for this mean; however, mouse models with different *K. pneumoniae* strains in some mouse models have proven difficult in research especially due to that depending on the *K. pneumoniae* strain the bacterium seems to cause variable severity of infection[175]. However, strain HS11286 used in this study was shown by parallel work done in lab 212 at the University of Leicester to be problematic in causing mortality in animal models. With the difficulty with mouse models and the rising cost of these models, coupled with the many ethical dilemmas behind their use, an insect model that closely resemble the mammalian model was chosen.

Galleria mellonella is one such a model. The immune system of these insects provides them with a strong antimicrobial protection by mounting cellular and humoral responses that mirrors that of mammalian hosts and thus can provide information that would also be provided from mammalian models [176, 177]. In numerous studies *Galleria mellonella* models has been shown to be often in line with mammalian models [178, 179]. This method however is time consuming as establishing controlled experiments can take time (establishing consistency with repeated infections with wild-type bacteria that has consistent results alongside a blank control injection).

A first step was establishing the wild type strain' virulence parameters and determining the dose of bacteria that is able to kill 50% of the insects. This dose was then used as a reference point to compare with the mutants constructed within the same background (the wild type strain HS04160, and then in HS11286 for the second set of experiments). Each of the four mutants was tested for virulence in this killing assay, and the results are shown in (figure 4.6). Tables 4.1 and 4.2 show the statistical analysis of these assays, and show a numerical value for the attenuation levels. For the assays shown in figure 4.6, almost 40% of the population injected with the KpGI-2 mutant survived as compared to wild type background strain. Very close levels of attenuation were

observed in the mutants for the *fic-rl* (almost 28% of insect population survived by day 5*)* and *docKp* genes (almost where almost 25% of the population remained by day 5). Deletion of the *fic-gn* (ORF-5 on KpGI-2) showed the least attenuation (15% survived by day 5), suggesting that ORF 1-4 are probably more related to virulence than the Fic gene coding for Fic protein on ORF5 of KpGI-2. It is noteworthy to mentioned that these larvae would be injected with the dose that is 10 times more concentrated than the LD50, leading to all larvae being dead by the fifth day, this accentuates any attenuation that may result. Survival does not indicate that the larvae are healthy by day 5, but rather, the surviving larvae, although still show signs of survival mentioned in the methods section (e.g. they respond to blunt physical stimulus), they however often look sickly (dark pigment on the surface, reduced movement) by the end of the day 5.

A



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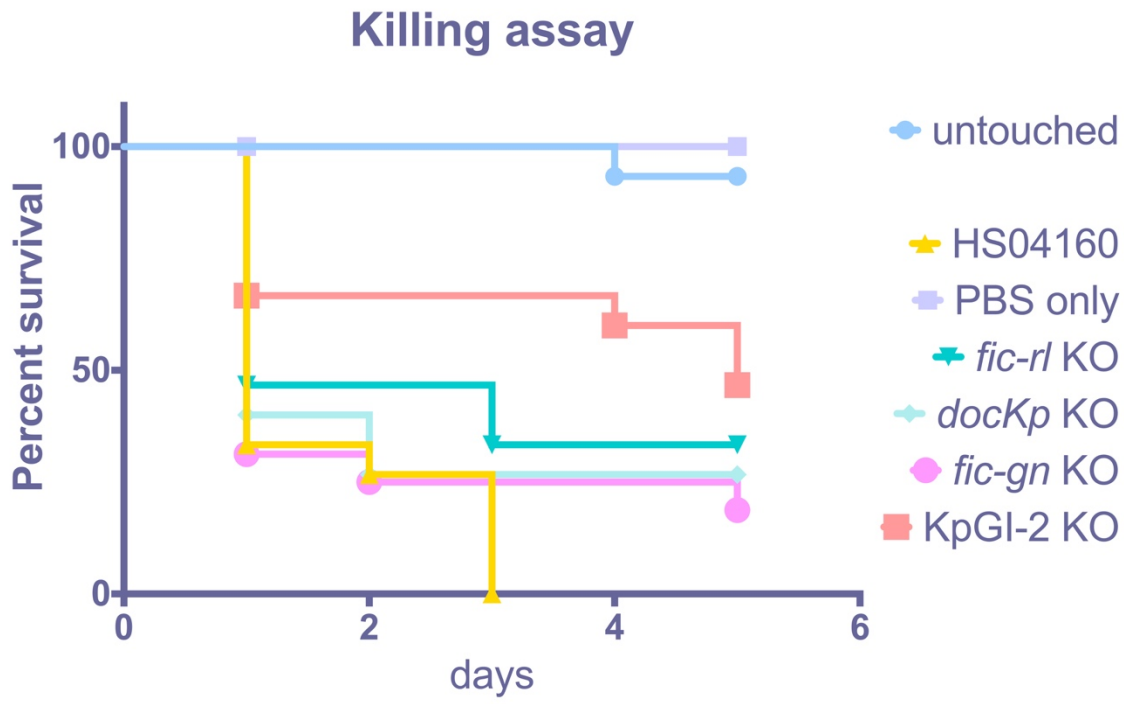

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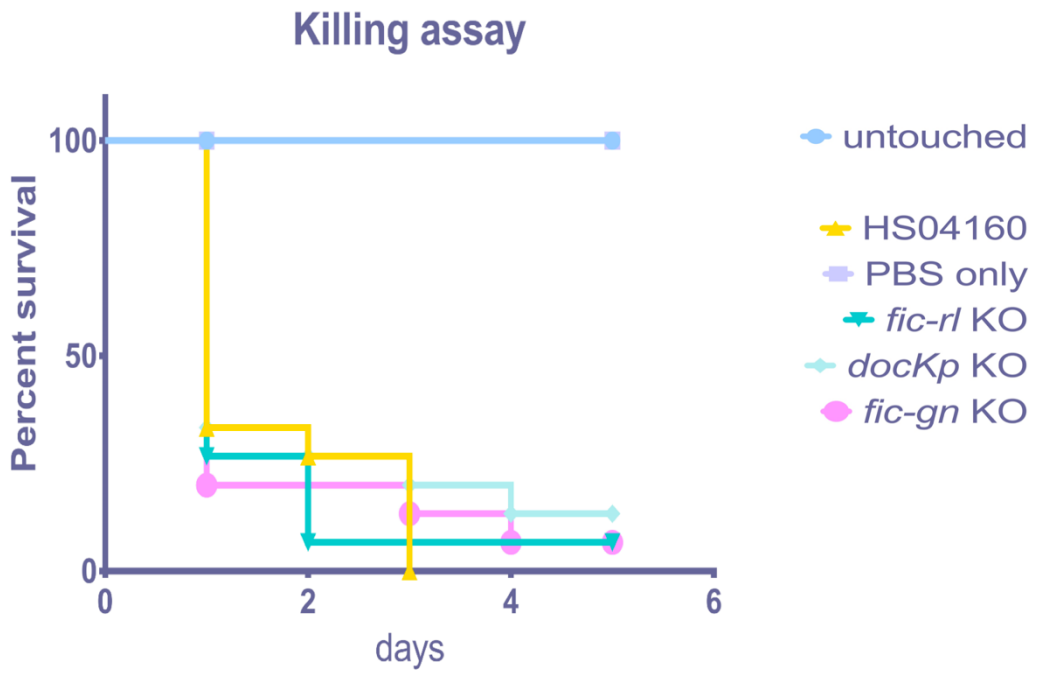


Figure 4-6 Assessment of *Galleria mellonella* killing in *K. pneumoniae* HS04160. In (A) representative image of the killing assay outcome determine the lethal dose needed to kill 50% of the larvae from a starting 0.2 OD600 culture, as described in methods section. The doses of the wild type strains are shown for different dilutions, starting from undiluted of an absorbance of 0.2 at OD600nm culture and using 3 dilutions of that: 1/10, 1/100, 1/1000. A dilution of 1/100 –light green- is the lethal killing dose

necessary to kill half the infected larvae (LD50). (B) A representative figure of the killing assays for each of the single mutants using the concentraion that was 10-fold higher that the LD50 determined for the WT strain (1/10 dilution of the measured 0.2 absorbance) Group sizes for each experiment consisted of 15 larvae. The death is consistent with results observed in control experiments where by the 3rd day most of the larvae were dead. The KpGI-2 mutant showed the most attenuation where almost 40% of the infected larvae survived by the end of the experiment. *fic-rl* mutants also showed significant attenuation (28 %) of the infected larvae survived by the end of the 5 days. Comparable results were also obtained with the *docKp* mutant, were almost a quarter of the infected larvae population survived at the end of the 5 days. The *fic-gn* mutant showed the least attenuation (15%) as compared to the 40% when the entire island is deleted. In (C) each mutant strain received the deleted gene carried on the vector pWSK29-Apra, constructed in chapter 3, to trans-compliment the deleted gene. The complementary gene and was put under the *lacZ* promoter and induced for 4 hour priors to preparing the inoculum, as mentioned in the methods section. The letter C in parenthesis after each plotted line indicates that the strain was trans-complemented. KpGI-2 Island was too difficult to clone into a vector (6.3 kb in size) and thus was not included. CFU counting was done for each injection to check for consistency of the amount of bacteria in each injection prior to each replicate test (not shown). The experiment was replicated 3 times and a representative experiment is shown in this figure, this shows that the deletion of these Fic genes caused an attenuation of killing of wax moth larvae which is partially complemented when reintroducing each deleted gene by means of vector carrying each gene.

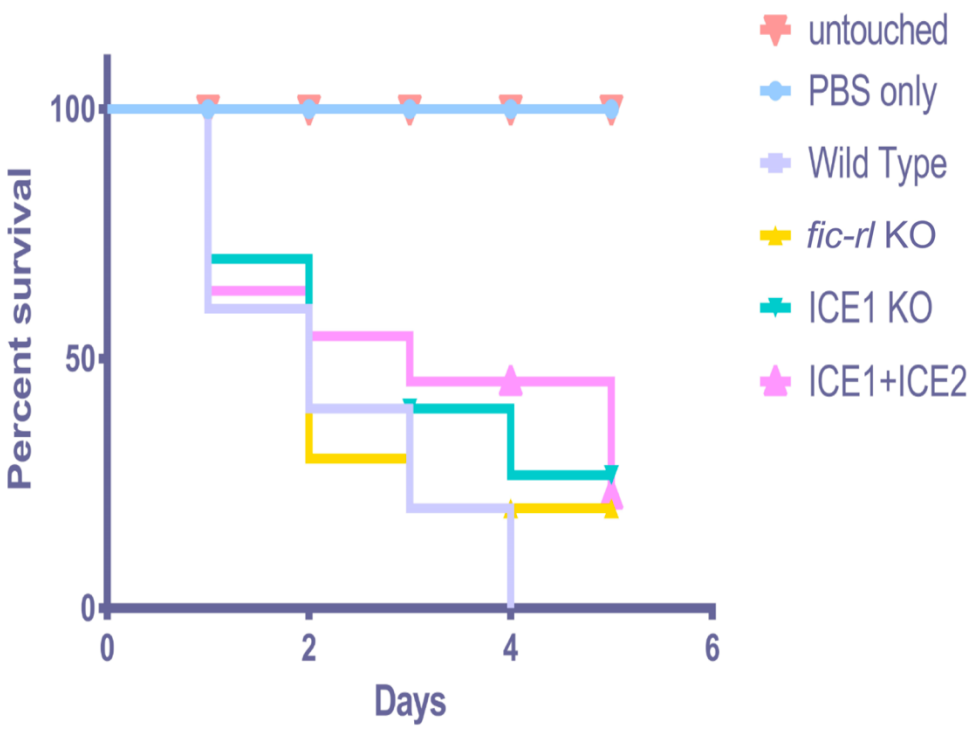


Figure 4-7 *Galleria mellonella* killing assay by different *K. pneumoniae* strain in background strain HS11286. The LD50 for HS11286 was determined similarly to the methods shown in 4.6 A (group sizes of 15 larvae as well). Following the determination of the LD50 the dose that was 10-fold higher than the LD50 was used similar to experiment shown in 4.6 B. The wild type strain is the HS11286 strain that was used to construct the ICE1 and ICE2 mutants (integrative and conjugative elements 1 and 2 that house the machinery for two distinct types of Type 4 Secretion systems) ICE1+ICE2 is a double mutant with the two elements removed. Following determining the secretion system responsible for secreting the effector Fic-RL, another mutant was constructed in the same background (Δ *fic-rl).* The results of the assay determine that the attenuation of the effector and the secretion system responsible for secreting it were very similar (16 % and 23 % infected larvae survived by day 5, respectively). The double mutant showed attenuation levels that resemble those seen in the ICE1 mutant. CFU counting was done for each dose to check for consistency of amount of bacteria in each injection prior to each replicate test.

Table 4-1 statistical analysis using two-way ANOVA, Bonferroni method to calculate the adjusted p-value for each set of survival assays done in HS04160. The table indicates the value that it obtained by comparing the survival of the wild-type versus each of the mutants shown by day 5. (n=15) data shown is for the three replicates of these killing assays.

Comparison HS0460 VS.	Mean percentage of WT survived by day 5	Mean percentage of mutant survived by day 5	Significant?	Adjusted p-value
Δ *fic-rl*	0.0	28.83	Yes	<0.0001
Δ docKp	0.0	24.4	Yes	<0.0001
Δ fic-gn	0.0	15.53	Yes	0.0146
Δ KpGI-2	0.0	39.96	Yes	<0.0001

Table 4-2 statistical analysis using two-way ANOVA, Bonferroni method to calculate the adjusted p-value for each set of survival assays done in HS11286. The table indicates the value that it obtained by comparing the survival of the wild-type versus each of the mutants shown by day 5. (n=15) data shown is for the three replicates of these killing assays.

Comparison HS11286 VS.	Mean percentage of WT survived by day 5	Mean percentage of mutant survived by day 5	Significant?	Adjusted p-value
Δ *fic-rl*	0.0	16.67	Yes	0.0421
Δ ICE1	0.0	23.33	Yes	0.0007
Δ ICE1 and ΔICE2	0.0	20	Yes	0.0060

Following the determination of the attenuation levels caused by each of the single gene deletions of the 3 genes coding for Fic proteins in HS04160 (*fic-rl*, *docKp* and *fic-gn* alongside the deletion of the island KpGI-2), a trans complementation experiment was done using the same parameters and methods as the described experiment in figure 4.6 B and using the same LD50 from 4.6 A. In this set of killing assays each of the mutant strains were complemented with the corresponding pWSK29-apra based construct, (described in chapter 3) that carry the complete coding frame for the genes *fic-rl*, *docKp* and *fic-gn* respectively, KpGI-2 was not included as it proved very difficult to clone into a plasmid probably due to the large size of the insert (6.3 kb). The experiments showed a partial complementation as follows:

- The *fic-rl* mutant killed close to 72% of the insects and the trans complimented mutant killed close to 92%), achieving roughly 78% complementation (or increase in virulence).
- The *docKp* mutant killed close to 75% of the insects and the trans complimented mutant killed close to 88%) achieving roughly 85% complementation (or increase in virulence).
- The *fic-gn* mutant killed close to 85% of the insects and the trans complimented mutant killed close to 89%) achieving roughly 95% complementation (or increase in virulence).

*the complementation percentage was calculated as: %of larvae killed by mutant/%of larvae killed by trans-complemented mutant (wild type killed 100% by end of day 5).
Plasmid carried apramycin resistance to select for the bacterial colonies harboring this plasmid, plasmid stability is expected to be reduced after being injected into the larvae as there is no apramycin added into the larvae.

Experiments described in Chapter 3, show that the secretion system present on ICE1 was responsible for the secretion of the Fic-RL protein in strain HS11286 background. A killing assay was done in this strain to determine the relationship between the virulence levels of mutants lacking the T4SS present on ICE1 (and a double mutant of ICE1 and ICE2) to the killing ability level caused by the deletion of the gene coding for the effector protein (Fic-RL) alone.

Strain HS11286 ability to kill the *G. mellonella* insects was much less than that of HS04160 (as evident by the LD50). The LD50 was determined to be a 1/10 dilution of a 0.6 OD @ 600nm culture. The larvae were injected with 20-fold of the LD50 (20 µl injections instead of 10 µl injections to cause death for all larvae by day 3 for all larvae injected) similar to was done in the previous experiment shown in figure 4.6. As shown in figure 4.7, the deletion of the secretion system on ICE1 (which was determined to be used in the secretion of the Fic-RL protein) and the double mutant of ICE1 and ICE2, caused an attenuation level that was very comparable to that caused by the deletion of the *fic-rl* gene.

These results show that the Fic homologs have variable effects on the virulence of *K. pneumoniae*, however, they demonstrate an important role of Fic-RL on the ability of this bacteria to cause infection and result in death of infected larvae.

4.3.5 Construction of pRK5mycBased Eukaryotic Expression Vectors

The pRK5myc plasmid, which is a eukaryotic expression vector with a strong CMV promoter, was used to clone 5 identified Fic homolog genes into the multiple cloning site of the plasmid. The CMV promoter in this plasmid enables strong expression of proteins inside human cells, once the construct is introduced by means of transfection. Once the vector carrying the gene of interest (as shown in figure 4.9) is delivered inside the human cells (as is described in the methods section) the CMV promoter initiates expression of a fusion protein with a MYC tag in the C-terminus of the protein (figure 4.8).

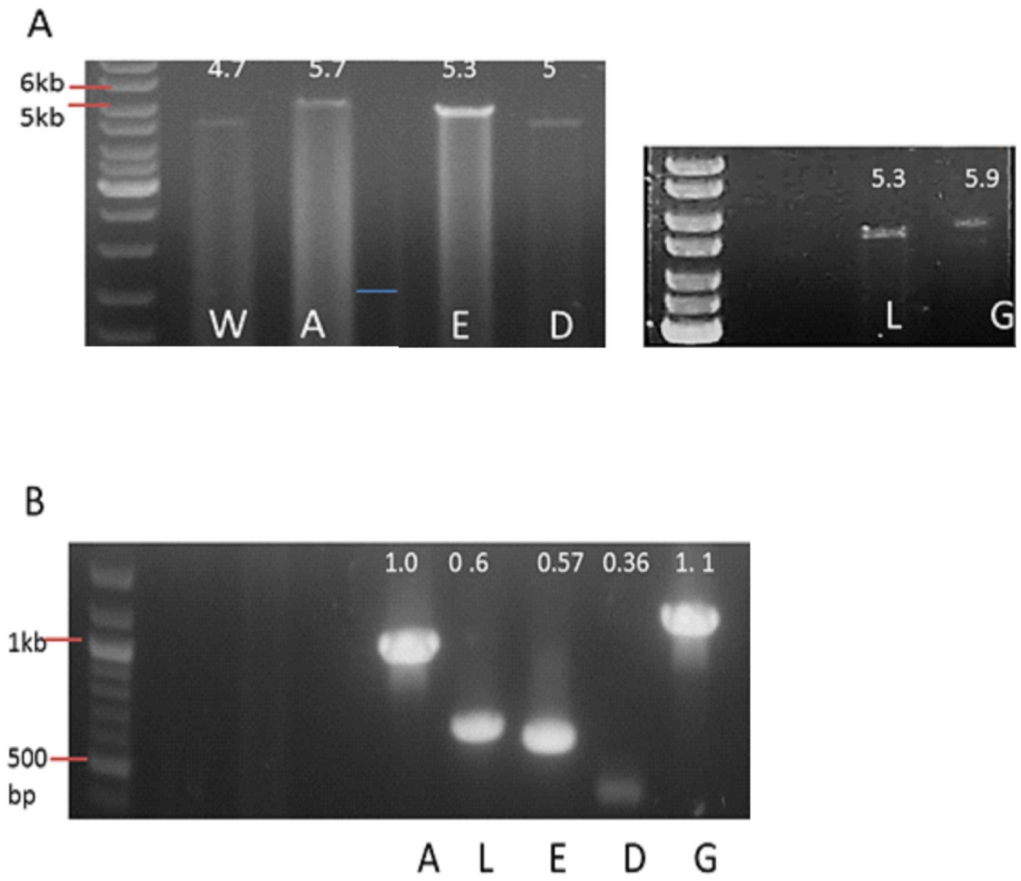
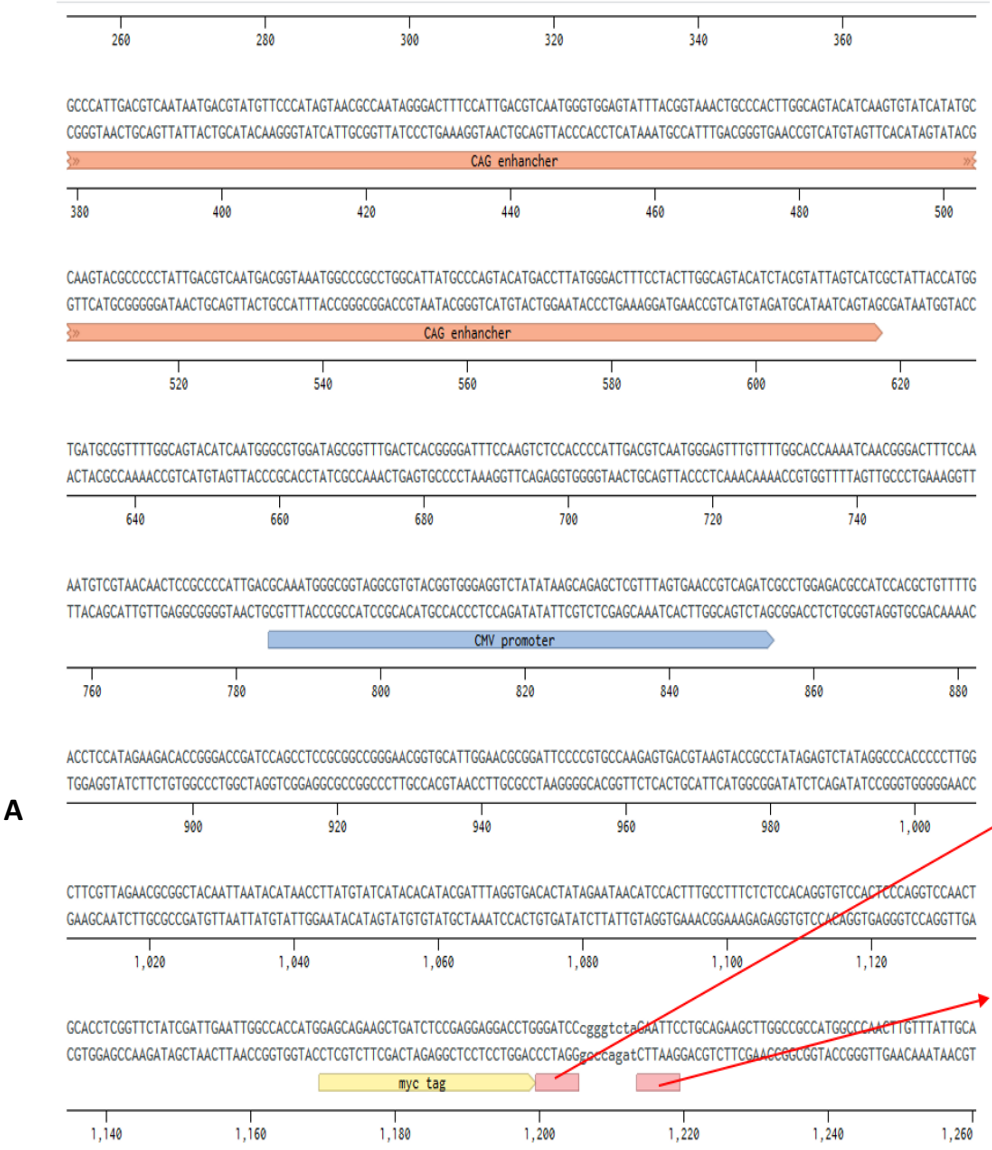


Figure 4-8 Gel electrophoresis analysis of pRK5myc constructs.

In (A) single enzyme digest (using EcoRI) of the constructed pRK5myc plasmids showing expected size band for each of the linearized plasmids (B) PCR amplification of pRK5-Myc plasmid carrying the insert in the finished plasmid to verify its presence and size within the multiple cloning region. W=wild type, A=fic-ad clone, L=fic-rl clone, E=fic-re clone, D=docKp clone, G=fic-gn clone. Expected size bands shown in estimates in kb on top of each lane.



A

BamHI cut site (GGATC(

EcoRI cut site (GAATTC)

B

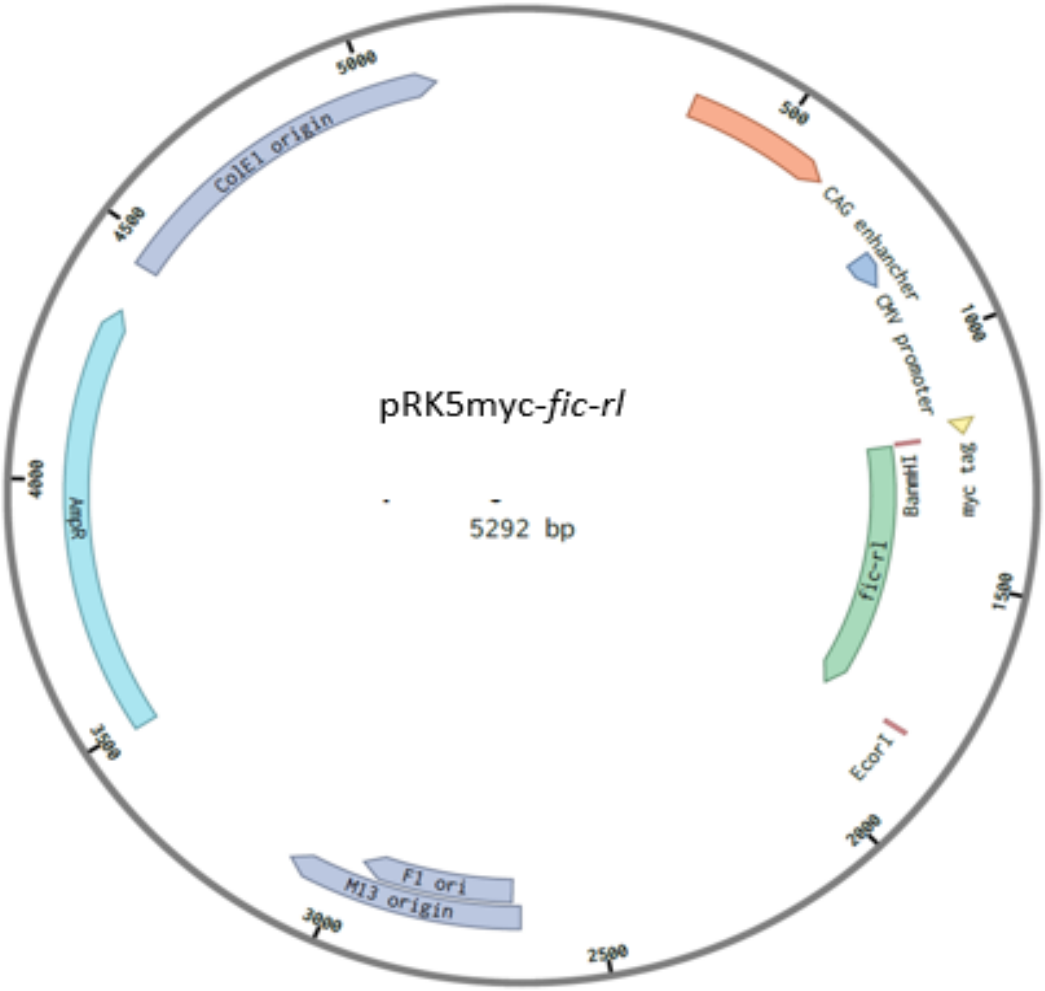


Figure 4-9, pRK5-myc constructs with Fic genes cloned into the MCS, showing plasmid maps and sequence. In (A) a part of the pRKY5myc plasmid, particularly around the multiple cloning site, showing the CMV promoter and the myc tag which would be translated into the C terminus of the resulting protein. The EcoRI site is inframe to the translation. (B) Plasmid map of one of the pRK5myc vectors constructed with the *fic-rl* gene inserted into the MCS using the indicated restriction enzymes as a representative map of these plasmids after the cloning process. Sequence for the mutant *fic-rl* gene (in which the sequence for conserved histidine was replaced with a sequence for alanine) is described in the methods section and in more detail in chapter 5.

4.3.6 Transfection and immunohistochemistry

Two cell lines were utilized in this section of the project, HK-2 human kidney proximal tubular epithelial cells and A549 a human lung mesothelioma epithelial cell line. These epithelial cell lines were selected based on the literature data reporting the assessment of toxins affecting the cytoskeleton by other Fic-domain bearing proteins. Transfection approach allows visualization of the effect of these proteins on the cytoskeleton [43, 44]. Moreover, both of these cell lines are epithelial in origin and enable the viewing of the cytoskeletal architecture more easily, so any adverse effects on the cytoskeleton would be more readily detected.

The transfection reagent Lipofectamine (Life Technologies) is able to bind DNA in lipophilic manner allowing cells that are conditioned with low serum media to take up these fat particles that carry the DNA with them. Once plasmids are inside the cells the CMV promoter is activated with resulting expression of the downstream gene allowing protein expression within the target cells. Transfected cells are then assessed using immunohistochemistry techniques, and imaged using EPI fluorescence and confocal microscopy (as described in the methods section).

The initial experiment involved transfection of plasmids carrying each of the five Fic encoding genes identified in *K. pneumoniae* followed by an assessment using fluorescence microscopy. This was aimed to determine which of the five proteins causes any effect (if any) on the cytoskeleton of these epithelial cells. HK-2 which are immortalized Human Kidney proximal tubular epithelial cells, were used for this purpose as they were readily available.

Following an overnight incubation, cells transfected with the pRK5myc-*fic-rl* construct were clearly affected. Actin staining in cells transfected with pRK5myc-*fic-rl,* and not the other 5 plasmids (nor the empty plasmid) was either absent or markedly reduced, as judged by the loss of the vibrant red color observed in non-transfected or normal cells. Cells transfected with other Fic domain plasmid constructs showed little to no

observable effect on the stress fibers morphology (figure 4.10). The small effects on the cytoskeleton of these cells could be attributed to the toxic effect of the transfection reagent (observed on the non-transfected cells as well exposed to transfection reagent alone).

A longer duration incubation of 72 hours resulted in a more marked effect on cells transfected with the construct allowing expression of Fic-RL protein. Cell to cell adhesion, stress fiber formation and overall cell size were all affected. The cells showed a marked reduction in size and exhibited the presence of large vacuoles. The actin filaments visualization was also impaired, and at default exposure level with 100 times increase of exposure with fluorescent microscopy setting resulted in visualization of an actin structure devoid of the normally observed fibrillary structure of these elements (figure 4.11). The transfection with construct encoding the other Fic domain bearing proteins resulted in an unclear phenotype where cells did not look as healthy as non-transfected cells; however, these cells maintained their stress fibers, and overall size and structure were similar to the non-transfected cells. These small variations can probably be attributed to cellular damage due to an overexpression of a foreign protein.

Following these initial findings, further characterization of the effect of the expression of the Fic-RL protein within eukaryotic cells was warranted. Another cell line of a different origin was used to verify the earlier observed effect on HK-2 cells and to try to observe any niche targeting by this protein to specific cells (possible predilection to one tissue type over another). In addition, to compare our findings to the observed phenotype of other FIC domain bearing proteins' function (e.g. VopS[39]), a mutant version of the *fic-rl* gene was constructed by switching the codon coding for hydrophilic conserved histidine in the signature motif with a codon coding for a hydrophobic alanine by point mutation of the nucleotide sequence, similar to the experiment involving the VopS protein [43]. Confocal imaging was utilized at this level (figure 4.11) and observed effects were conformant with those of VopS protein. A range of partial disruption of the stress fiber actin structure (membrane ruffling, nuclei fragmentation, decrease in total size) to all out collapse was observed in these instances. It is noteworthy to mention that cell shape was roughly estimated in confocal microscopy by the fact that cells that

were transfected with the wild type version of the protein required more plane sections that the mutant's version or the non-transfected version, denoting that the overall shape of the cell was more round (a rounded cell requires more cross sections across its depth than a normal shaped cell).

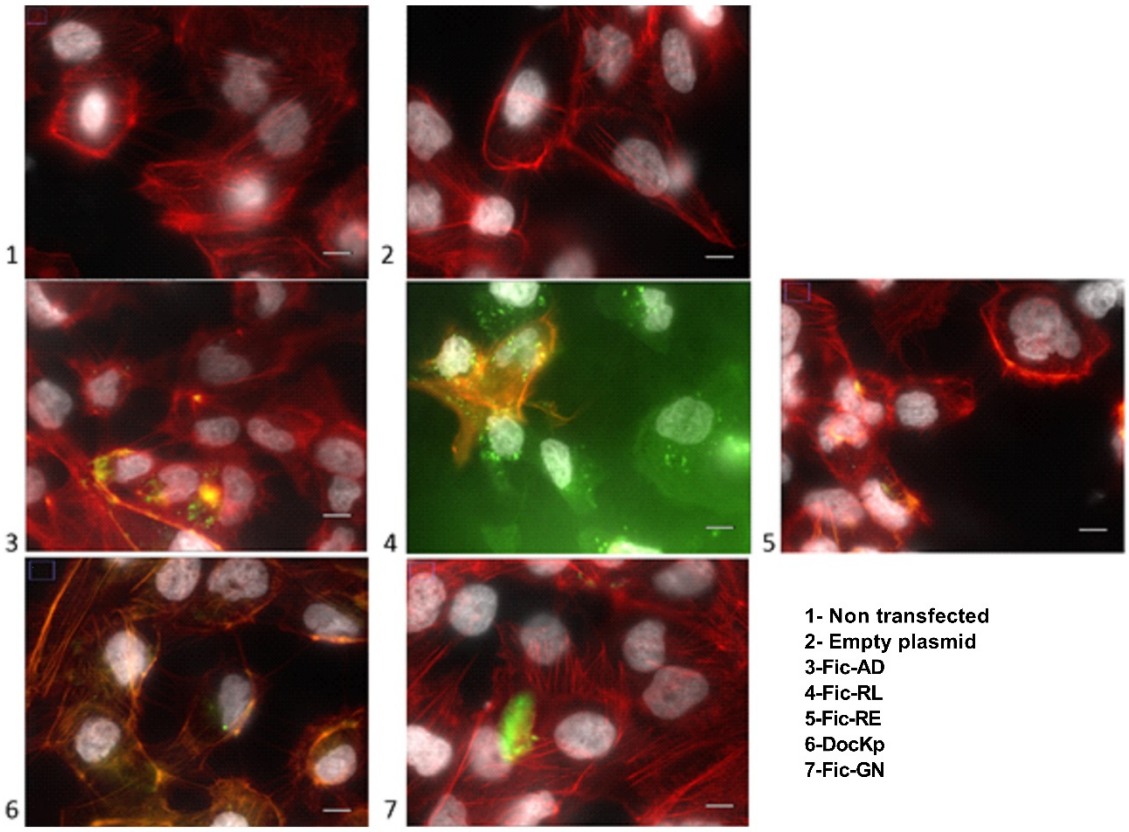


Figure 4-10 Immunofluorescence microscopy images of Human Kidney cells 2 (HK-2) cells transfected overnight with the pRK5-myc plasmid carrying each of the five Fic protein encoding genes identified in *Klebsiella pneumoniae.* Non-transfected cells appear in image 1, image 2 showed cells transfected with an empty plasmid, images 3, 4, 5, 6, and 7 (FIC-AD, FIC-RL, FIC-RE, FIC-DOC AND FIC-GN in order) show the five FIC domain bearing proteins tagged with a myc tag and visualized by using antibodies to the myc tag carrying an Alexa 488 green fluorochrome. Cells showing the green staining are cells that have been transfected and expressing the tagged protein. Scale

bar is shown at bottom right corner of each image (25um), these images are representative of the effect observed.

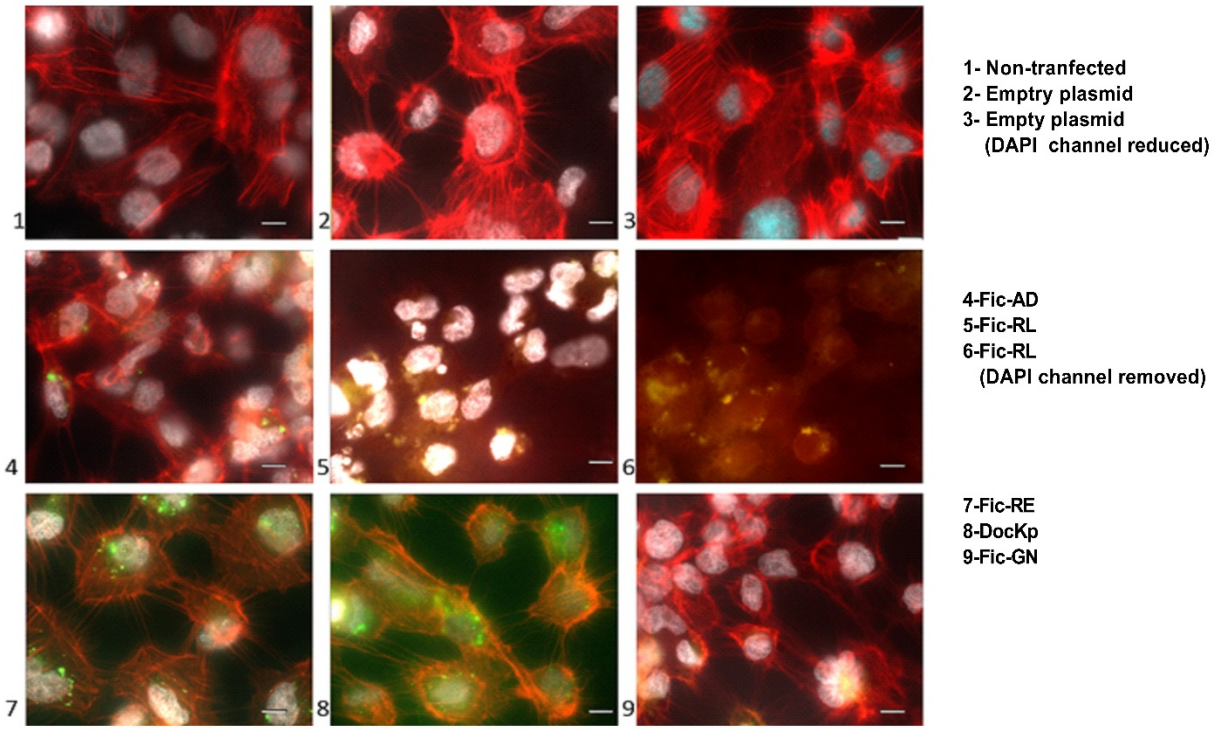


Figure 4-11 Immunofluorescence microscopy images of Human Kidney cells 2 (HK-2) cells transfected for 72 hours with the pRK5-myc plasmid carrying each of the five FIC encoding genes identified in *Klebsiella pneumoniae.* Non transfected cells appear in image 1, image 2 and 3 show cells transfected with an empty plasmid, images 4, 5, 7, 8, and 9 (FIC-AD, FIC-RL, FIC-RE, FIC-DOC AND FIC-GN in order) show the five FIC domain bearing proteins tagged with a myc tag and visualized by using antibodies to the myc tag carrying an Alexa 488 green fluorochrome, as in the previous image. Image 6 is a replication of image 5, however, with the removal of the DAPI channel that shows the nuclei in white. This shows the degree of the effect of the protein on the cells that is partially masked by the nucleic dye(DAPI). Scale bar is shown at bottom right corner of each image (25um), images shown are representative of the effect observed.

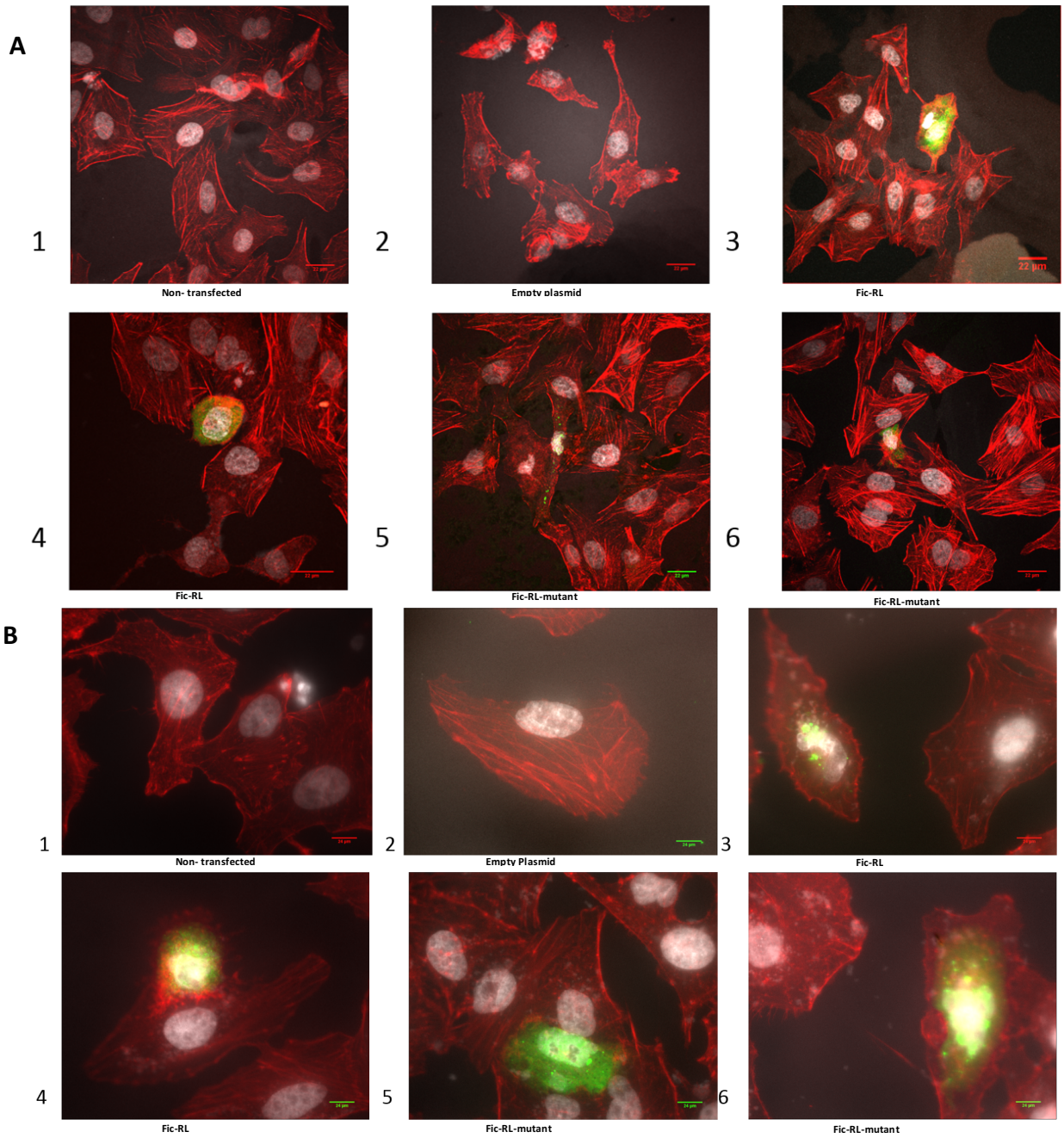


Figure 4-12 Confocal and fluorescence microscopy images of Human A 549 cells transfected with the pRK5-myc plasmid carrying *fic-rl* gene and mutated form of *fic-rl* gene obtained in *Klebsiella pneumoniae.* (4.12.A) confocal images of the transfected A549 human lung mesothelioma cell line. Image 1 shows the un-transfected cell, while image 2 shows cells transfected with an empty plasmid, image 3 and 4 show cells transfected with the wild type version of the *fic-rl* gene. In image 3, cells show membrane ruffling and still maintain some form of stress fiber structure; while in image 4, possibly a cell that was transfected longer show more cell architectural collapse and rounding. Images 5 and 6 show cells transfected with the mutated

version of the protein. Overall, such cells show a structure that is similar to surrounding healthy cells. (B) is a replication of the images (same order) in (A) however, they are taken using regular fluorescence microscope. The effects of membrane ruffling and actin destabilization are more evident in image 3; whereas image 4 shows a similarly rounded and collapsed cell much like in the confocal image. In images 5 and 6 cells transfected with the mutant gene show similar appearance that is seen in the confocal images in (A).

4.4 Discussion

The secretion assay performed in chapter 2 revealed that only Fic-RL of the five Fic homologs is secreted by the T4SS encoded on the integrative element (ICE1). The nature of such a possible effector was assumed to be a toxin, as delivery of a protein into the inside of the host cell by a specialized secretion system is one utilized method of introducing toxin proteins into target cells.

To test this theory, an infection test was done using the insect *Galleria mellonella* model. These insects' immune system provides them with strong antimicrobial protection by mounting cellular and humoral responses that mirrors that of mammalian models[180] and thus can provide information that can be gained from animal models especially in light of the supporting evidence that show it to be in line with mammalian models [176, 178, 179, 181]. However, this model has its limitation in that the immune response mounted by the larvae is not as complex as seen in mouse models.

The first set of experiments focused on establishing if any of the *fic* genes, once deleted out of the genome, would cause a measurable attenuation. The deletion of kpGI-2 island caused a relatively high attenuation (close to 40% of the larvae survive by day 5 as opposed to none that were injected with the wild type bacteria) but not due to the deletion of the gene that codes for the Fic protein (*fic-gn*) present in the ORF5 of the island, as the deletion of that gene alone cause a moderate attenuation (close to a 15% of larvae survive as opposed to none in ones injected with the wild type bacteria). These results suggest that other elements present on the KpGI-2 island play a synergistic or perhaps an additive effect with the Fic protein coded in its last reading frame (genomic islands tend to carry genes with similar functions such as pathogenicity genes, fitness genes, antimicrobial resistance...etc.[104, 172, 173]).

The data from the infection model show that the single gene deletion that caused the highest level of attenuation is that caused by the deletion of the *fic-rl* gene (the gene that was shown by Bioinformatic analysis to be universally preserved in *Klebsiella* species), the deletion of this gene caused nearly 28% of the larvae to survive an infection

with the mutant strain, to the end of the experiment as opposed to none in the wild type bacteria. This information coupled with the information from chapter 2 (Fic-RL was the only one shown to be a secreted effector), strengthened the theory that this is indeed a toxic protein involved in virulence.

To further tie the secretion system to its effector, a second set of experiments was done using strain HS11286 background and a set of mutants generated by another study in our lab (deletion of T4SS in either ICE1 or ICE2). Deleting the *fic-rl* gene in this strain enabled us to help tie the secretion system to its effector. The deletion of the effector or the ICE1 element showed comparable attenuation levels (where almost 30-35% of insects survived the experiment as opposed to none of the ones injected with wild type bacteria).

An interesting result however, was that the deletion of the toxin part of the toxin-antitoxin system (*docKp* of the Doc-PHD system) showed comparable attenuation levels to the deletion of the *fic-rl* gene. This may potentially be explained by new reports that show that bacteria use this toxin to switch itself to a more resistant form[171]. This data supports the notion that some of the toxin anti-toxin system play a role in virulence as perhaps a mechanism to escape the hosts defenses until the bacteria is present in a more suitable environment within the host to exert its effects (until it is internalized within the hosts cells for example). However, this theory needs further testing and could shed more light on the lifecycle of *K. pneumoniae* as an intracellular pathogen.

After determining the ability of bacteria to secrete these proteins by T4SS, understanding the effects that Fc-RL exerts once it is inside the eukaryotic cell was the next goal, especially with support from the attenuation observed in the infection model. To study these effects, genes encoding Fic domain proteins from *Klebsiella* were cloned into a Eukaryotic expression vector (pRK5-myc). This plasmid is commonly used and found in literature and is able to transiently transfect many cell lines, while utilizing a strong viral (CMV) promoter to drive recombinant protein expression [118, 182].

Most Fic domain bearing proteins that caused direct cytotoxicity exert their action by targeting protein of the Rho family of GTPases which causes actin cytoskeleton to collapse and ultimately lead to cell death. The Five homolog genes coding for the 5 Fic domain bearing proteins in *K. pneumoniae* were transfected into HK-2 using the pRK5myc vectors and immunohistochemistry utilizing fluorescent microscopy was used to determine which of these proteins caused effects on the cytoskeleton of HK-2 human kidney cells.

Stress fiber formation in non-muscle cells, which is regulated by Rho family of GTPases, is important for vital cell functions such as migration, adhesion and signaling[94] [57].The observed cytotoxic effects of Fic-RL on the cytoskeleton are similar to those described in literature caused by other Fic domain bearing proteins (VopS , IbpA) once introduced within the target cell. Fic-RL similar to these proteins lead to cytotoxic effects on the actin cytoskeleton structure and resulted in collapse and cell death[94] [58]. When the transfection was allowed to run for a duration of 72 hours the effect of Fic-RL was more pronounced (this allowed all cells in the culture to reach a full cell cycle and express the protein).

Transfection and successful protein expression in these images (figures 4.9 and 4.10) was of different success levels (although the amount of transfection reagent, amount of DNA and cell cultures were all of equal level), this could be attributed to stability of the proteins or that cells at different stages of the cell cycle take up the DNA-lipid complexes and results in different levels of expression of protein. However, the Fic-RL images shows the most prominent effect on the cytoskeleton (complete collapse, cell rounding). The other Fic proteins (DocKp, Fic-GN, Fic-RE and Fic-AD) had not caused such an effect, where the cytoskeleton of cells expressing these proteins preserved its fibrillary structure, with evident cell-to-cell interaction and preservation of overall structure of the cell. However, some cells exhibit signs of cytotoxicity (especially in the 72 hour sets and not in the 24 hour sets) which can be attributed to starvation of cells as these cells are already starved prior to transfection to enhance the take up of DNA lipid complexes (lipofectamine reagent can also be a cause of cell toxicity).

An effect shown in the 72 hour experiment where the surrounding cells that seem to have no Fic-RL protein expressed also exhibit similar levels of cytotoxicity and cell death (this is not seen in the other Fic protein for the same timeline), no evidence can be given to the cause of this effect, but it is a possible consequence to the cell death of surrounding cells. However, fact that this only happened in the culture of cells expressing Fic-RL protein hints that this protein might have a role in this phenomenon.

Further confirmation of the cytotoxic effect caused by Fic-RL was done on a different cell line (A549 human mesothelioma alveolar cells) and utilized a mutated version of Fic-RL alongside the wild type form (the mutant version had the conserved histidine in the FIC motif replaced with an alanine). Confocal and fluorescent microscopy were used and images showed that the actin cytoskeleton is more or less preserved when the mutated version of Fic-RL is expressed within the cell but when the wild type form is expressed evident disruption and eventual collapse is shown (the more protein is expressed the more the effect is evident). Fluorescent and confocal images also show a dose response effect, when a smaller amount of expressed Fic-RL protein is shown, cytotoxicity is less pronounced (membrane ruffling is shown, less rounding of the cell), whereas cells showing a larger amount of expressed Fic-RL protein exhibit actin cytoskeleton collapse and rounding of the cell.

To conclude the results so far: Fic-RL protein coded by a conserved gene in *K. pneumoniae* is secreted by T4SS present on ICE1 in these bacteria. Fic-RL is likely to be an effector that, once delivered inside the host cell, it has been observed that intracellular activities of this protein (as assessed by the expression of the protein following transfection), results in a prominent effect on the host cell cytoskeleton. Furthermore, mutant strains that lacked the gene coding for Fic-RL showed a relatively significant attenuation as compared to the wild type that was partially complemented, once the deleted gene was reintroduced by means of a plasmid construct.

Parallel work done by my collaborator (Robeena Farazand-Lab212, III department at the University of Leicester), has focused on the conjugation ability of *K. pneumoniae.* Using mutant strains (deletion of ICE1, ICE2, double mutant of ICE1 and ICE2, as well

as mob1 gene-DNA mobilization gene used for secretion of DNA used by T4SS on ICE1- and mob2 -DNA mobilization gene used for secretion of DNA used by T4SS on ICE2-). The results generated by that study complemented the results of this one (shown in the table below).

Table 4-3 collection of results on assays performed on several mutants of T4SS in *K. pneumoniae.* Assays include, killing assay using *Galleria mellonella*, secretion analysis of the Fic-RL protein and conjugation frequency analysis.

Strain	WT HS11286	Δ*fic-rl*	ΔICE1	ΔICE2	Double mutant(ΔICE1, ΔICE2)	Δ*mob1*	Δ*mob2*
Percentage survived by day 5 of survival experiment	0	16.67	23.33	43.33	20	0	40
Able to secrete effector Fic-RL	Yes	N/A	No	Yes	No	Yes	Yes
HGT (conjugation) frequencies	8.6×10^{-6}	NA	Below the limit of detection ($<1\times 10^{-8}$)	2.1×10^{-6}	Below the limit of detection ($<1\times 10^{-8}$)	Below the limit of detection ($<1\times 10^{-8}$)	1.8×10^{-6}

Conjugation frequency was non-detectable for the ΔICE1 and the double mutant strains, which are the same strains that were unable to secrete Fic-RL. Both these strains showed similar levels of attenuation in the killing assay. The deletion of the *mob* genes, genes related to the mobilization of DNA for secretion (in T4SS), did not lose the ability to secrete Fic-RL protein, and was in fact an expected result seeing as their genes are involved in DNA transfer and not protein transfer.

Chapter 5

Determining Enzymatic Activity of the Fic-RL Protein

5.1 Background

The first mention of Fic domain was in an article by Kawamukai et al where they reported a gene in *E. coli* that when mutated caused a filamentous-like growth of this bacterium upon addition of cyclic AMP [36]. The function of this domain, however, remained elusive until several secreted bacterial effectors were identified which carry this domain like VopS [43] and AnkX [42]. The method used to determine the enzymatic activity of the Fic domain bearing proteins involved the identification of the target of few of these Fic domain bearing proteins and by observing the phenotypic changes in human cells elicited when these proteins were introduced into the human cells. One of the effects observed was the cytoskeletal changes (cell rounding, collapse) that following introduction of Fic proteins in to human cells, which suggested an interference with the function of the Rho GTPases (such as Rac and Cdc42). Thus, these potential target proteins were assessed for modifications after interactions with of the Fic domain bearing proteins [39, 42, 43, 183]. Another phenotype observed (besides cell rounding and collapse) was the ability of bacterial pathogens possessing Fic proteins to escape host defences by maintaining intracellular vacuoles or diverting cellular traffic. This suggested the targeting of RAB family of GTPases. Further research in this area revealed that some Fic proteins promote intracellular infection by hindering the transfer of vesicles along cytoskeletal routes by hijacking the control of RAB GTPases and disrupting downstream signaling [40, 45, 55].

In the case of Fic proteins that target Rho GTPases, the first description of this function was done for the VopS protein [43]. Yarborough et al [43] incubated the Fic domain bearing protein VopS with its proposed target Rac. This resulted in a molecular weight increase in Rac following incubation that corresponds to the addition of an AMP residue (adenosine 5’ monophosphate). Such enzymatic activity has been referred to as AMPylation ever since. Further research in the same study revealed more information which later proved to be consistent among Fic proteins. It was observed that introduction of a mutation resulting in changing the invariant histidine in the FIC motif of VopS to alanine resulted in a protein acting as a substrate trapping substance to GTP-loaded Rac (it would bind only GTP-loaded Rac without releasing it). Furthermore, the

wild-type VopS, and not the mutant, caused Rac to not be able to bind to one of it's the downstream effector, PAK [43].

As Fic domain bearing proteins are gathering more attention, they are also being further characterised. This has allowed researchers to note that all the characterised Fic domain-bearing proteins studied so far function by post-translational modification of their target proteins. Fic proteins modify cytoskeletal regulatory elements, mainly Rho[42] and RAB GTPases, by the addition of a substrate that incorporates a phosphate group in its structure and other nucleoside monophosphates (such as AMP or UMP[39, 43]), Phosphocholine [40, 45] and even a phosphate group alone [38, 46](as a substrate for phosphorylation [46] as described in [41]). This posttranslational modification usually targets a specific recipient site in the target protein, which might vary depending on the Fic protein. Substrates utilised by the FIC motif are all diphosphate molecules. After catalyses by the FIC Motif, one of the two resultant phosphates from the phosphate-containing molecule is transferred to the target amino acid on the target protein. It has been postulated that the orientation of the binding between the Fic protein and the substrate determines which of the two resulting molecules of the diphosphate moiety is used for the reaction (for example if ATP is used, the reaction would either use AMP or phosphate as the result of the breaking of the ATP molecule)[41]. This post-translational modification usually targets Tyrosine, Threonine or Serine amino acids (all hydroxyl containing amino acids) on their target protein, completing the picture of the substrate and target of the proteins so far [45, 47, 48].

It is noteworthy that described bacterial Fic domain bearing proteins seem to be mainly targeting GTPases, such as Rho, RAB and EF-Tu (Elongation Factor Tu or EF-Tu which is considered a GTPase and is targeted by the doc toxin, a Fic protein. [38, 46]).
In addition to the secreted virulence factors, many toxins in a toxin-antitoxin (TA) scheme have been shown to utilise the Fic domain, such as the Doc-PHD system [38] or VbhT-VbhA system[94].

5.2 Aims

The aim of this part of the project is to provide information on the enzymatic activity of the Fic-RL protein and the target of this activity. A recombinant wild-type protein (Fic-RL) was produced and purified to assist in this process. A mutant version of the Fic-RL (Fic-RL-HA) protein was also utilised to verify whether any observed activity is dependent on the conserved FIC motif (HPHXXGNG in *K. pneumoniae*). The nucleotide sequence has been mutated to replace the codon for the invariant conserved Histidine in the amino acid sequence with an amino acid of opposite polarity (the non-polar Alanine). The target proteins of the Rho family of GTPases were chosen due to the observed phenotype of the cytoskeleton of human cells that were described in chapter 4 (collapse, membrane ruffling). This phenotype gives the suspicion that the target is possibly a member of the Rho family of GTPases, as conformant to other Fic domain bearing proteins described in the literature.

5.3 Results

5.3.1 Constructing vectors

5.3.1.1 Bioinformatics

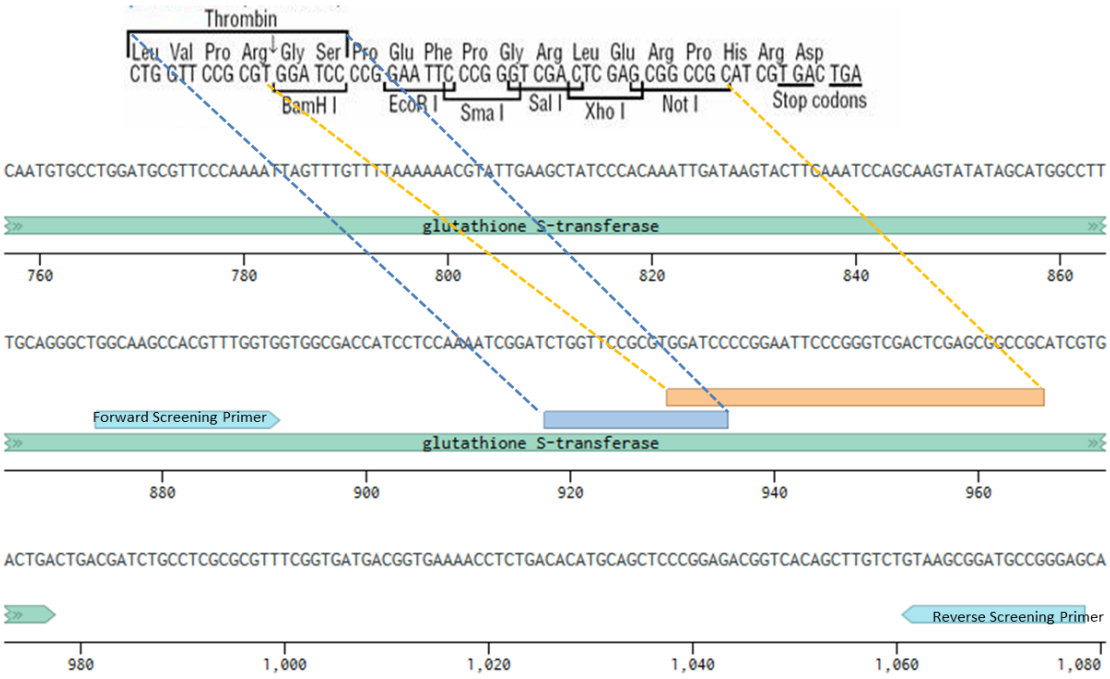
The sequence for the *fic-rl* gene was obtained from the NCBI database from the genomic sequence of the strain HS11286 (Locus tag: KPHS_48980). ApE plasmid editor and Benchling online tool were used to visualise the sequence and annotate open reading frames and identify the multiple cloning sites (MCS) of the pGEX4T-1 plasmid. The sequence of *fic-rl* gene was assessed to determine which restriction enzymes can be used for cloning experiments (recognition sites for which are present in the MCS and which do not have cut sites on the gene sequence (described in 5.1.3.1)). BamHI and EcoRI restriction enzymes were selected; these two recognition sites are present in the MCS in a forward direction (Figure 5.1).

5.3.1.2 Mutant insert construction

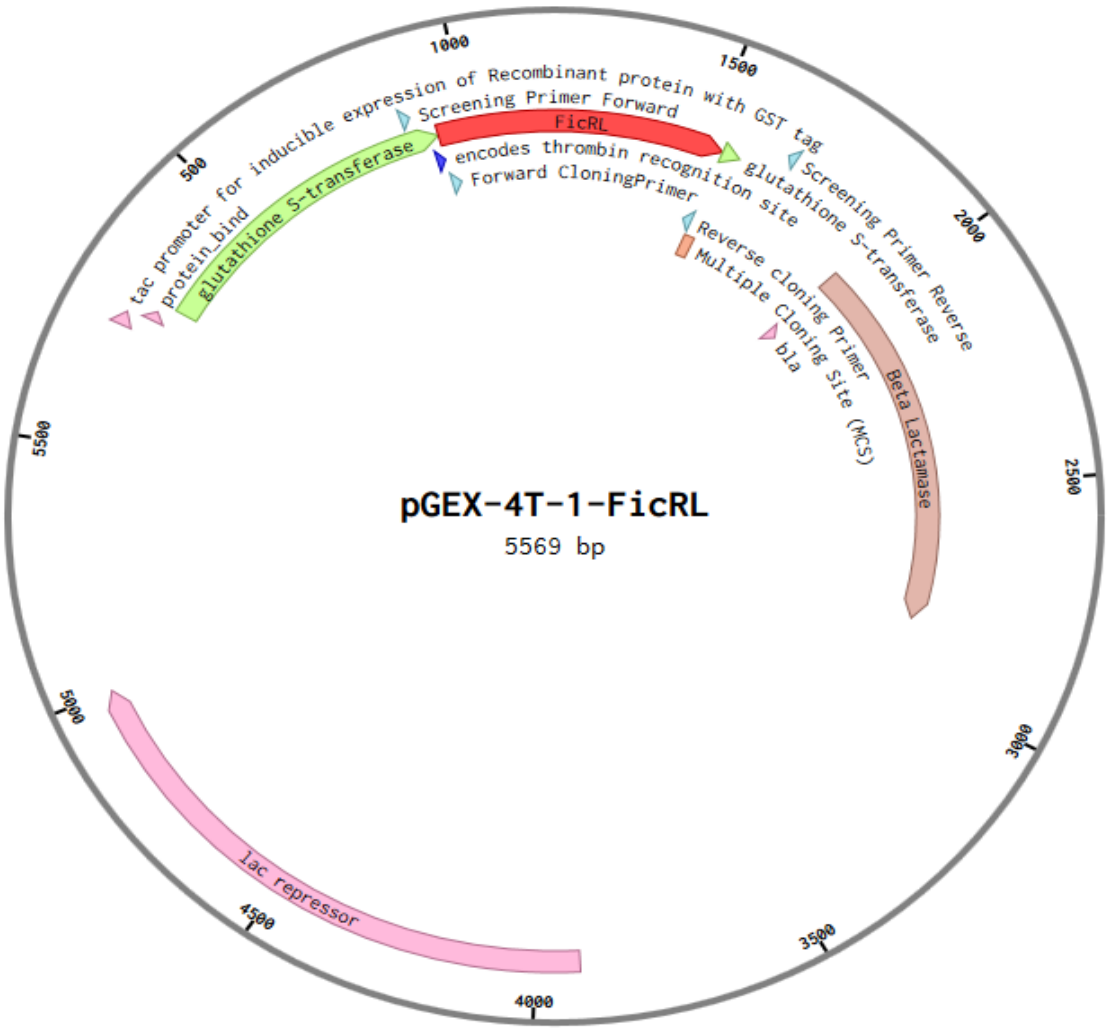
The aim was to introduce point mutations to a DNA template obtained from the wild type HS11286 strain. These point mutations aimed to change the codon sequence of the invariant or conserved Histidine in the FIC motif (**H**PHRLGNG), which is a polar amino acid, to a codon sequence coding for a nonpolar amino acid (Alanine) with a resultant amino acid sequence in the conserved FIC motif of (**A**PHRLGNG). A switch in polarity at this place in the amino acid sequence has been shown in multiple studies to cause a loss of function for the Fic protein in question.

Identifying the exact sequence for the target Histidine residue to be mutated was done by obtaining an amino acid translation of the DNA sequence of the *fic-rl* gene obtained from NCBI. By using the Expasy translation tool to obtain the amino acid sequence, the three nucleotide sequence location was identified (Figure 5.2). The wild-type sequence of CAC at location 399-401 (counting from the start codon sequence of the 603 bp sequence) was intended to be changed to a GCC (figure 5.2). The primers used to create the two segments with the point mutations are shown in Figure 5.1-C. The forward cloning primer (purple) is paired with the reverse SOE primer (orange) while the forward SOE primer (grey) is paired with the reverse cloning primer at the 3’ end (purple) to obtain two fragments with complementary homologous sequence the size of the SOE primers. The two segments are later joined using SOE PCR as described in the methods section to obtain the completed sequence (Figure 5.2).

A


Thrombin
Leu Val Pro Arg Gly Ser Pro Glu Phe Pro Gly Arg Leu Glu Arg Pro His Arg Asp
CTG GTT CCG CGT GGA TCC CCG GAA TTC CCG GGT CGA CTC GAG CGG CCG CAT CGT GAC TGA
BamH I
EcoR I
Sma I
Sal I
Xho I
Not I
Stop codons
CAATGTGCCTGGATGCGTTCCCAAAATTAGTTTGTTTTAAAAAACGTATTGAAGCTATCCCACAAATTGATAAGTACTTGAAATCCAGCAAGTATATAGCATGGCCTT
glutathione S-transferase
760
780
800
820
840
860
TGCAGGGCTGGCAAGCCACGTTTGGTGGTGGCGACCATCCTCCAAAATCGGATCTGGTTCCGCGTGGATCCCCGGAATTCCCGGGTCGACTCGAGCGGCCGCATCGTG
Forward Screening Primer
glutathione S-transferase
880
900
920
940
960
ACTGACTGACGATCTGCCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCA
Reverse Screening Primer
980
1,000
1,020
1,040
1,060
1,080

B


pGEX-4T-1-FicRL
5569 bp
tac promoter for inducible expression of Recombinant protein with GST tag
protein_bind
glutathione S-transferase
Screening Primer Forward
FicRL
encodes thrombin recognition site
Forward CloningPrimer
glutathione S-transferase
Screening Primer Reverse
Reverse Cloning Primer
Multiple Cloning Site (MCS)
bla
Beta Lactamase
lac repressor
500
1000
1500
2000
2500
3000
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4000
4500
5000
5500

C

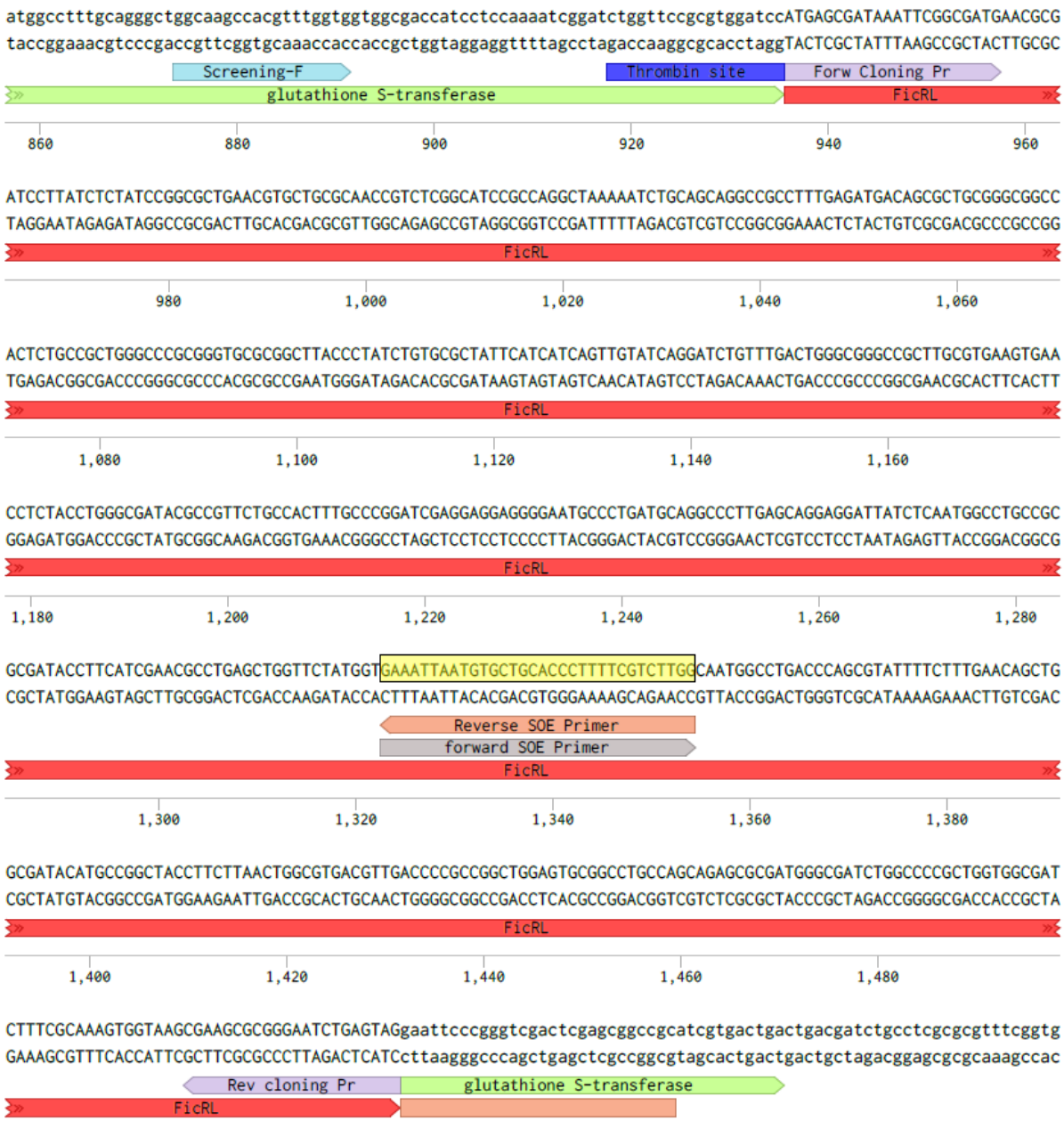


Figure 5-1 Protein expression of Fic-Rl and Mutant Fic-RL using pGEX4T-1 vector 5.1.A This figure shows a section in the sequence of the pGEX-4T-1 plasmid (empty plasmid); the specific segment highlights the multiple cloning site(orange), the thrombin recognition site (dark blue) and the forward and reverse screening primers (light blue) all within the sequence of GST(green). The reverse primer is outside the sequence of GST, the restriction sites, stop codons and thrombin recognition sequence are shown above in black with the codon frame translation to prevent any frameshifts. In 5.1-B, a map of the vector pGEX-4T-1 with the *fic-rl* gene inserted into the MCS is shown. Arrows indicate open reading frames and the direction of the arrow indicates start to stop codon read (blunt end is start codon and arrow end indicates the stop codon for

the ORF). The mutant version of *fic-rl* was similarly cloned using the mutated insert prepared with SOE PCR.in 5.1-C the completed sequence of the vector with the *fic-rl* gene inserted is shown, the SOE primers used to create the mutant sequence of *Fic-rl* are highlighted in the middle of the sequence.

5.3.1.3 Cloning procedure of pGEX-4T-1-FicRL and pGEX-4T-1-FicRL-HA

Plasmid pGEX-4T-1, a commonly used vector for protein expression, was used to clone the gene sequence for *fic-rl* gene and the mutant gene sequence (*fic-rl-ha*) with an aim to produce the proteins Fic-RL and Fic-RL-HA figure 5.1-B. The *fic-rl* insert was produced by amplifying the *Fic-rl* gene from genomic DNA prepared from strain HS11286 (which was also used to prepare the mutant insert). Vector pGEX-4T-1 has a sequence encoding Glutathione-S-Transferase (GST) tag with an MCS at the tailing end of the GST sequence that allows the integration of sequences into the MCS. Once the desired sequence was cloned into the plasmid, a fusion gene created with a GST tag sequence inserted at the 5’ end of the gene, which would be expressed at the N-terminus side of the resulting protein. Resultant recombinant protein should be about 26kDa larger as to account for the size of the 211 amino acids of the GST tag (figure 5.1-B). Restriction enzyme cutting, ligation and transformation were all done as mentioned in the methods section. Results of the cloning procedure and sequencing are shown in figure 5.3.

A

B

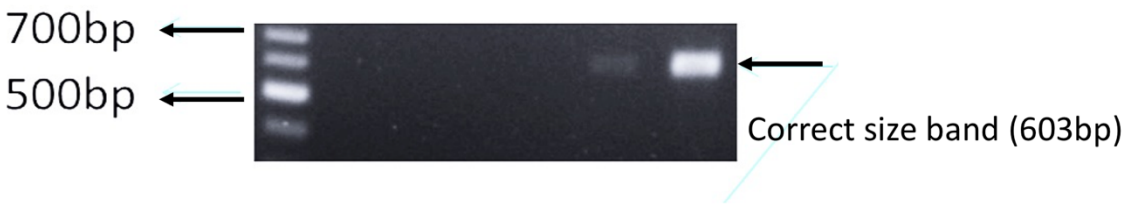
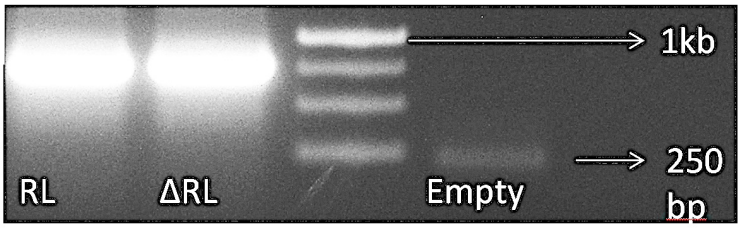


Figure 5-2 construction of mutant *fic-rl* gene. In 5.2-A the top portion of the figure shows translated amino acid sequence of the wild-type DNA sequence of the *fic-rl* gene highlighted in orange, while indicating the conserved Histidine the sequence with a red box. The bottom sequence shows the amino acid sequence of the translation of the mutated DNA sequence after two point mutations are introduced by means of primers also highlighted in orange, with the mutated amino acid (Histidine is mutated into an Alanine which is indicated with a red box). the two nucelotides that were mutated to induce this change are shown in the small DNA segment shown between the two amino acid translations, these two point mutations of the CA nucleotides shown in green in the wild-type sequence are changed in the primer below this sequence for a GC shown in red, which ultimately changes the codon to code for an Alanine instead of a Histidine. The bottom DNA sequence with the red highlighted GC is used as a primer (along with a reverse complement sequence) to produce the two fragments of DNA, that when joined together by SOE

PCR give the completed DNA fragment with the mutation introduced, a gel image of such successful SOE PCR amplified DNA fragment is shown in 5.2-B, the empty lanes represent unsuccessful SOE-PCR and the last lane on the right side of the image shows a bright correct size band for the SOE product, which is later confirmed in figure 5.3.B by means of sequencing.

A



B

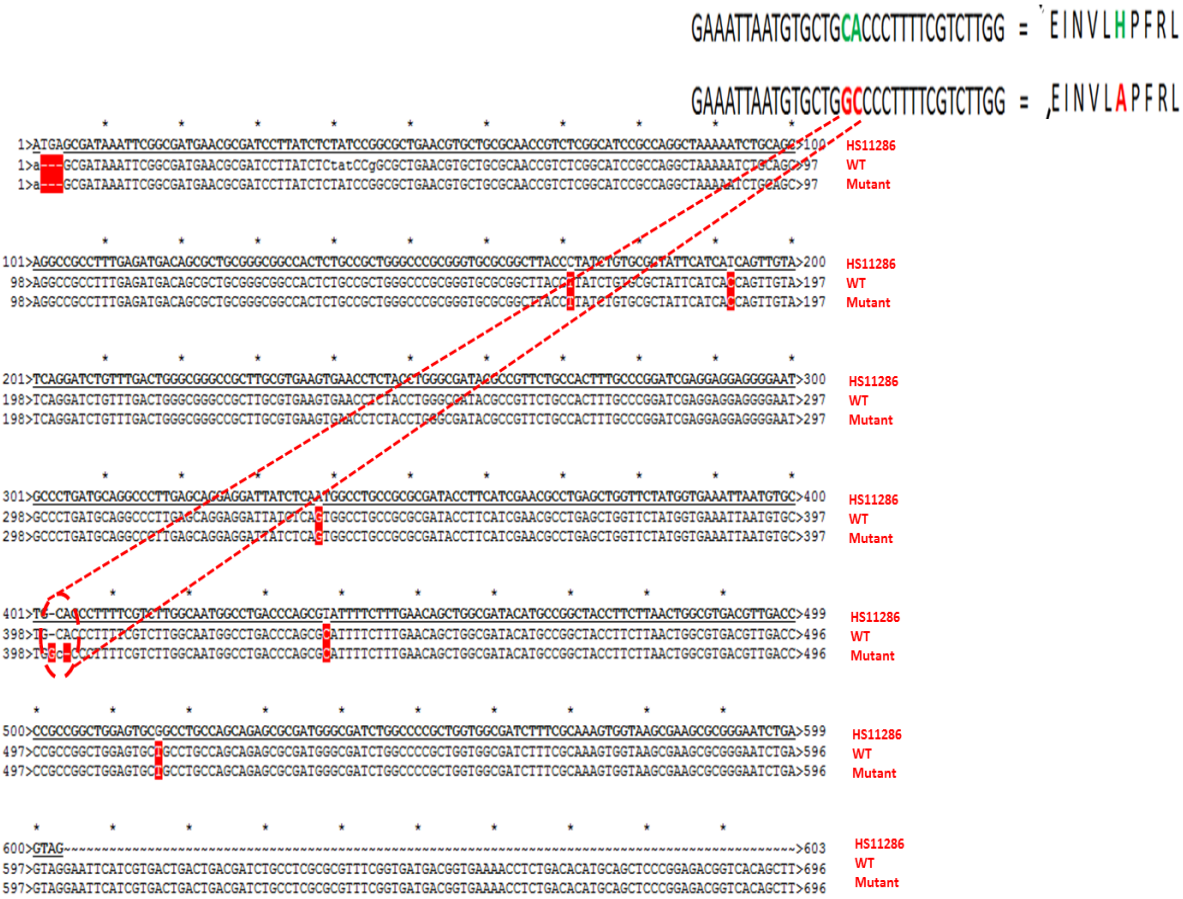


Figure 5-3, 5.3-A a gel electrophoresis analysis of PCR amplified DNA fragments of pGEX-4T-1 constructs. Gene *fic-rl* (lane labelled RL) and the mutant form of *fic-rl*

(labelled ΔRL) are shown with identical product sizes of ~805bp. Using the screening primers shown in Figure 5.1, PCR with an empty plasmid as a template would yield a product of ~208bp, shown in the lane on the right side of the DNA ladder. Sequencing of those two segments is shown in figure 5.3.B. In figure 5.3-B sequencing results of the two segments produced in figure 5.2-A as compared to the wild type sequence and the sequence of the HS11286 strain obtained from NCBI database. all three sequences are aligned together and indicated using the red font. HS11286 refers to the original sequence obtained from NCBI database. WT refers to the sequence of the segment of the wild-type gene of *fic-rl* inserted into pGEX-4T-1, while Mutant refers to the segment with the point mutation inserted into the vector produced in figure 5.2.A. These segments were obtained using external primers shown in Figure 5.1-A.

5.3.2 Protein expression

E. coli strain BL21 (DE3) was used as an expression host. Either plasmid pGEX-4T-1(*fic-rl*) or plasmid pGEX-4T-1(*fic-rl*-HA) were transformed into this host for expression purposes. Transformation was done as described in the materials and methods section, and colonies that exhibited the correct phenotype (resistance to Carbenicillin) were verified using colony PCR with the screening primers indicated in Figure 5.1.A in light blue colour.

Protein expression was first tested in 20 ml cultures growing over 6 hours (with induction with IPTG) in 50 ml conical flasks using multiple temperatures (37 ^{o} C, 25 ^{o} C and 17 ^{o} C). Pellets were collected and the bacteria were then lysed by boiling. The levels of the protein expression were screened using SDS-PAGE for the presence of a correct sized band that is present in the test sample but absent in the control (no plasmid added). The next phase was to verify the band as the protein in question, which was done by sequencing the band in question using MS/MS sequencing and verifying the sequence using NCBI Database.

Both sequences obtained were verified to be that of the Fic-RL protein and of the mutant version of the Fic-RL protein; it is noteworthy to mention that the expression temperature had a minor effect on the expression levels of the two proteins. However, multiple attempts at retrieving these proteins yielded wild-type protein concentration

(as measured by Nanodrop following elution) that was higher from the culture grown at 37 ^{o} C, whereas the mutant protein concentration retrieved was higher from the culture grown at 25 ^{o} C (figure 5.4).

Proteins expression on a larger scale was done in 500ml volume LB broth cultures in 2.5 L flasks grown overnight while being supplemented with appropriate antibiotics and IPTG inducing agent.

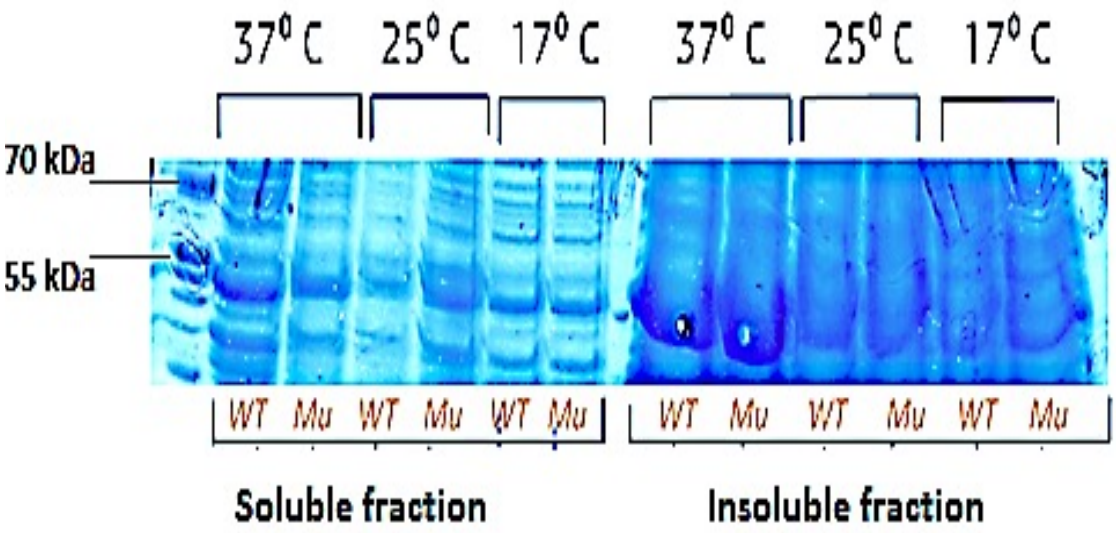


Figure 5-4 depicts an SDS-PAGE analysis of different samples from cultures grown at indicated temperatures, (Fic-RL-shown as WT, and the mutant version of Fic-RL shown as Mu). The sample loaded in each lane was prepared from a 20-ml culture after a 6-hour incubation at the temperature shown at the top of the gel. Soluble fraction refers to the protein extracted from the supernatant after sonication, whereas the insoluble fraction refers to the pellet remaining after sonication. This SDS-PAGE is a representative of three replicates; expression of Fic-RL was reproducibly higher at 37 ^{o} C whereas the mutant form of Fic-RL was reproducibly highest at 25 ^{o} C.

5.3.3 Protein purification

Following expression, proteins present in the soluble fraction were collected and purified using reduced glutathione beads, as mentioned in the methods sections. In short, the proteins that were expressed both contain a sequence for GST (Glutathione-S-transferase) which has a high affinity to the GSH (Glutathione Sepharose High-performance beads). The GSH beads were inserted into a column and the cell lysate -containing the GST-tagged proteins- was passed through the column. All proteins except those that contain the GST tag were washed away during the washing step. GST-tagged proteins bound to the column were eluted by reduced glutathione. The eluted proteins were collected and analysed by SDS-PAGE (shown in figure 5.5). The bands of interest were sequenced, using MS/MS sequencing service.

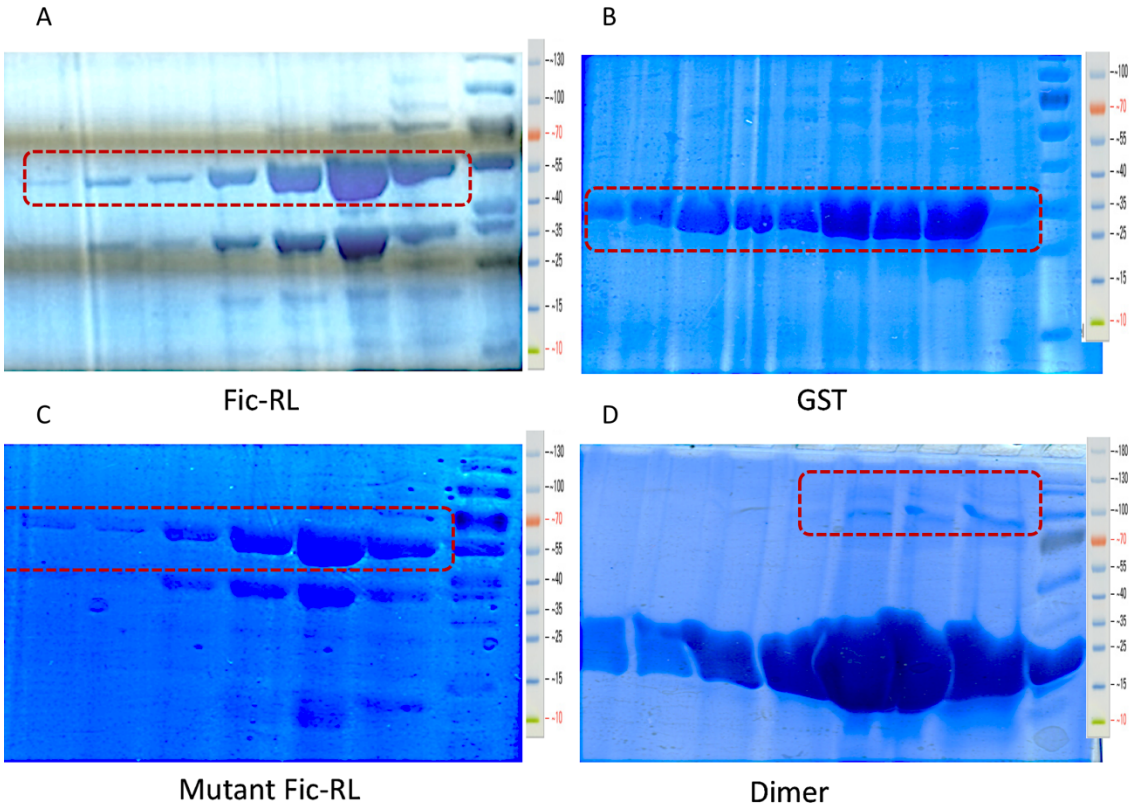


Figure 5-5 shows SDS-PAGE analysis of the eluted off the GST, Fic-RL and Mutant Fic-RL proteins after column purification and elution using reduced Glutathione. Usually, these proteins are fully eluted in the first three fractions (shown for each). In (B) GST

prepared from an empty plasmid is shown, (A) shows the Fic-RL protein elution fractions and in (D) shows a dimer for of the Fic-RL is clearly shown; this dimer is more prominently shown by boiling the samples for 1 minute instead of the usual five minutes (not shown). In (C), the mutant Fic-RL elution fractions are shown. Dimer for this protein is not apparent after 1 or 5-minute boiling (data not shown). The anticipated sizes are roughly 26 kDa for GST, 50 kDa for Fic-RL and mutant form of Fic-RL, and 73 kDa for the dimer form of Fic-RL.

5.3.4 Guanine Exchange Factor (GEF) assay

The wild type and a mutated FicRL proteins that were retrieved (alongside GST tag protein alone, which was produced using an empty p-GEX-4T-1 vector, the GST tag alone is used as a negative control) were used to determine the activity of these proteins. Commonly targeted members of the Rho-GTPase family, namely Cdc42, RhoA and Rac1, were used as substrates. The 3 proteins of the Rho-GTPase family mentioned remain the most studied members of this family and thus were chosen for this assay due to that and commercial availability of reagents. Previous experiments with other Fic domain bearing proteins have shown that one or more of these 3 proteins are targeted; however, the methods by which these proteins are targeted seems to differ [38, 39, 45, 55, 183].

Kits procured from Cytoskeleton Inc. (BK100) (for a diagram refer to Figure 5.9 A) were used to perform the enzymatic assay as described in the materials and methods section. The determination of the activity of these proteins relies on the principle based on fluorescent activity recorded when a fluorophore bound substrate is used or modified by the enzymatic reaction, namely that of a guanine exchange factor. The exchange of the GDP-bound Cdc42, Rac1 or RhoA for the GTP-bound form is enzymatically catalysed by Guanine Exchange Factors (GEF). A GEF would generate an active form of a Rho GTPase when it catalyses this exchange reaction resulting in the active GTP-bound form [184, 185]. A GEF enzyme would bind to a GDP-bound Rho GTPase and destabilise the

GTPase-GDP, releasing the GDP. In the presence of high ratios of GTP to GDP intracellularly (which is mimicked in this reaction by providing GTP substrate in abundance), the GEF enzyme would stabilise a GTP molecule to replace the released GDP in the GTPase complex[186]. A GEF is unable to maintain binding to GTPase-GTP complexes, resulting in the release of the GTPase-GTP complex from the GEF. In this case, the GTPase-GTP complex, when using the fluorophore bound GTP substrate would emit higher fluorescence. This outcome identifying if an enzyme was able to bind the inactive GTPase present in the wells (Cdc42-GDP or Rac1-GDP or RhoA-GDP) and catalyse the release of the GDP (thus working as a GEF). The binding of the GTP substrate to the now free GTPase, results in emitting fluorescence that is recorded, as mentioned in the methods section.

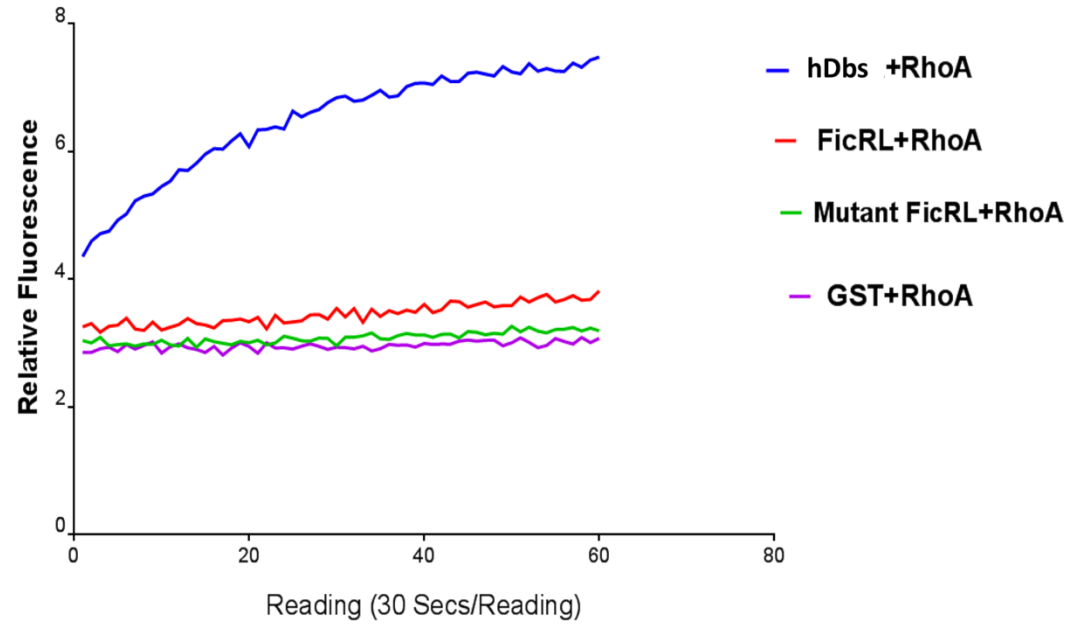
Results of an assay performed with hDbs, a known potent GEF, are shown in figures 5.6 and 5.7 (represented by the blue line). hDbs works as a positive control to this experiment. hDbs is clearly acting as a GEF, transferring the Guanosine monophosphate molecule to the provided Rho GTPase present in the reaction as the target for the activity (Cdc42, Rac1 or RhoA). The components for the reaction (Fluorophore bound substrate, GEF enzyme hDbs and target protein as Rac1, Cdc42 or RhoA) were all added together, and the fluorescence was measured starting from the first minute (time needed to transfer the 96 well plate vessel to the spectrophotometer). The fluorescence is recorded at regular intervals of 30 seconds, as ruled by the provided software for the Varioskan spectrophotometer.

In the case of RhoA, as shown in figure 5.6, a strong increase in the fluorescence intensity when the positive control hDbs was recorded over the 30-minute period (60 readings one every 30 seconds). The increase in relative fluorescence (RF) was almost 2-fold, indicating a positive utilisation of the substrate and thus activation of RhoA by exchange GDP for GTP. FicRL and the mutant version of FicRL both failed to illicit a similar increase and recorded fluorescence levels that are comparable to that of the negative control (GST tag alone). Multiple runs of the reaction were performed, and a representative record is shown in figure 5.6.

As for Rac1, the positive control itself is a weak activator, and any positive outcome should either show similar results to the positive control or exceed it. However, it was recorded on the multiple runs that neither of the two proteins (FicRL or the mutant version of FicRL) showed a positive GEF activity, and final readings were very close to the negative control and did not show a slow weak rise that is shown with the positive control.

A clear GEF activity of Fic-RL, however, was recorded in the case of Cdc42 as shown in figure 5.7. Multiple runs have confirmed that when the reaction is conducted in the presence of Cdc42 as the target Rho GTPase, FicRL (and not the mutant version of it), was able to activate Cdc42 to levels comparable to those of the positive control hDbs. An almost 2-fold increase in fluorescence intensity for FicRL was recorded over 60 minutes (120 readings) with almost a 2.5-fold increase for that of the strong activator hDbs protein.

These results suggest that FicRL protein can act a GEF for Cdc42, but not for RhoA or Rac1. Furthermore, a mutation of the conserved Histidine in the Fic motif sequence results in an inactive protein.



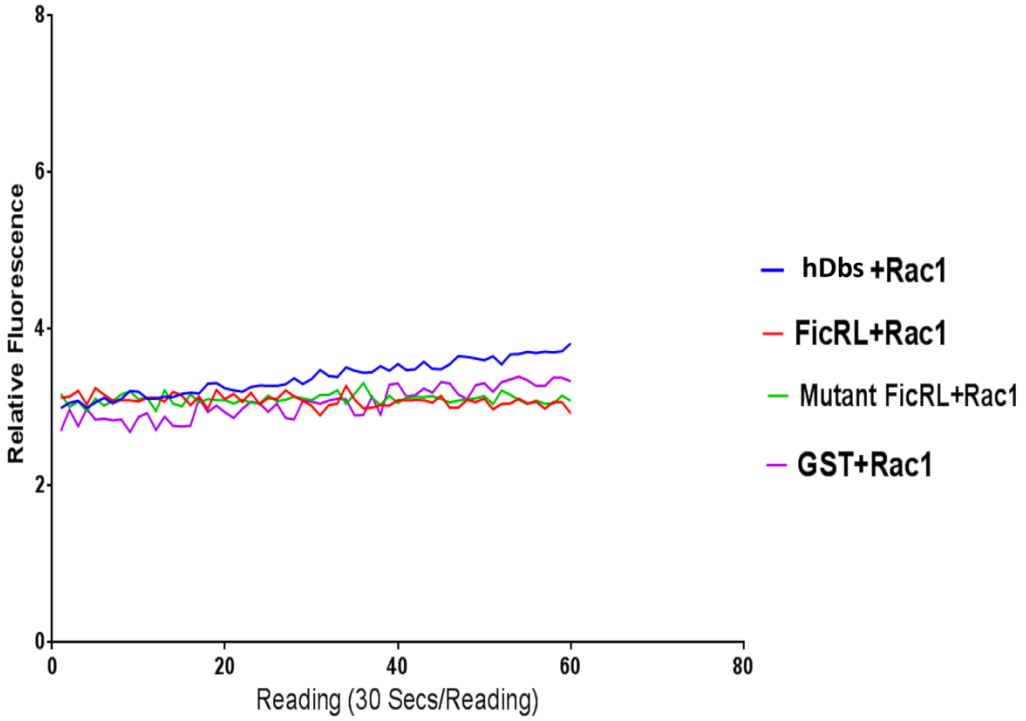


Figure 5-6 GEF exchange assay for Rac1 and RhoA, two of the three RhoGTPases tested. The above figure shows the exchange assay of RhoA, the bottom one shows that of Rac1. The positive control hDbs which is a strong activator (GEF) of RhoA and a weak activator for Rac1 is shown in blue for both figures. The negative control (GST

alone) that is expected to have no GEF activity is shown in purple, the wild type form of FicRL is shown in red while the activity of the mutant form of FicRL is shown in green. Same concentration of Fic-RL or mutant Fic-RL was added into each reaction, and a 10:1 ratio was used against the Rho GTPase tested (10 times the concentation of the RhoGTPase would be added of the Fic-RL or mutant Fic-RL) loaded into each well. The assay was allowed to run for the recommended 30-minute period. The above figure show a representative repeat of 3 independent Rac1 and RhoA assays.

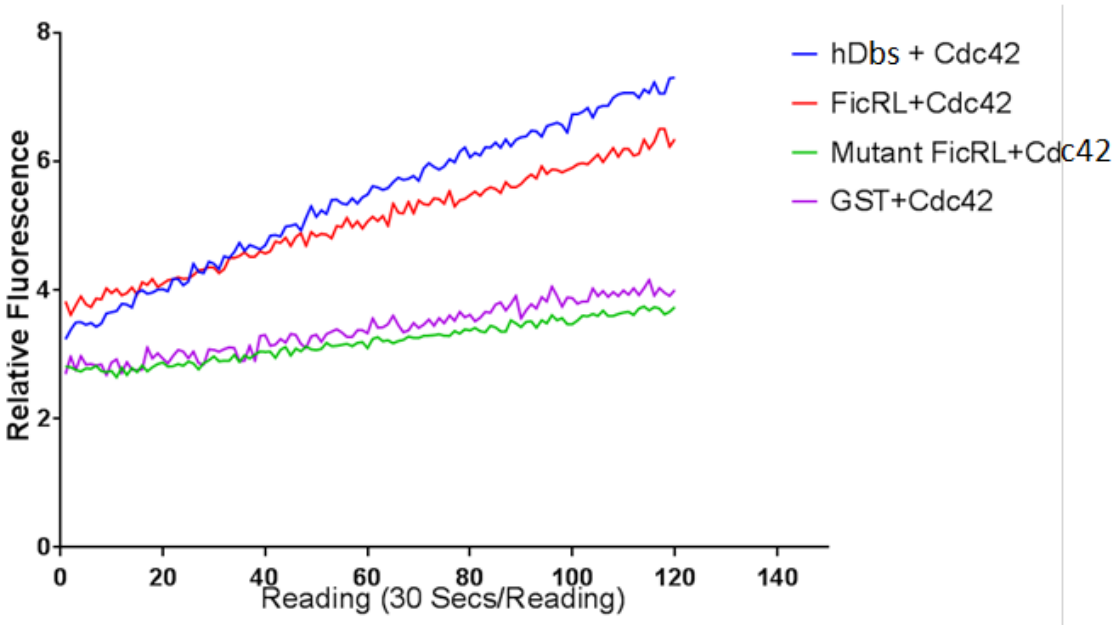


Figure 5-7 GEF exchange assay for the RhoGTPase Cdc42. The positive control hDbs, which is a strong activator (GEF) of Cdc42, is shown in blue, while the negative control (GST alone), which is expected to have no GEF activity is shown in purple. Same concentration of Fic-RL or mutant Fic-RL was added into each reaction, and a 10:1 ratio was used against the Cdc42 (10 times the concentration of the Cdc42would be added of the Fic-RL or mutant Fic-RL) loaded into each well. The wild type form of FicRL is shown in red, showing a fourfold increase in the fluorescence intensity while the activity of the mutant form of FicRL is shown in green, showing levels of intensity similar to that of the negative control (GST alone in purple). These reactions were allowed to exceed to 60 minutes of readings (120 reading) before terminating

recording. The figure shows a representative repeat of 3 independent assays using Cdc42.

5.4 Discussion

Assessment of the effects elicited by the expression of Fic-RL in human A549 lung epithelial cell line and HK-2 Human kidney cell line revealed an apparent impact on the cell cytoskeleton (membrane ruffling, cell rounding, eventual collapse of cell as evident in chapter 4 results). Mutant version of FicRL did not induce these cytoskeletal effects when it was similarly expressed in these cell lines. This finding when coupled with the knowledge of the role of the Cdc42/Rac-PAK pathway in the regulation of the actin cytoskeleton suggested that FicRL (but not a mutant version) affects the actin cytoskeleton, and the Rho family of GTPases could be the likely targets, as is the case for some of the other described Fic proteins such as VopS.

The Cdc42/Rac are the dominant regulatory elements for the activation of the PAK pathway (the main effector for these Rho GTPases), which in turn is responsible for the formation of actin structures such as filopodia, lamellipodia, membrane ruffles, focal adhesion complexes/remodelling and stress fibres[71, 76]. Results in this chapter clearly demonstrate that FicRL can act as a GEF for Cdc42. As FicRL had a direct effect on one of two dominant activators of the PAK pathway (Cdc42), thus FicRL's effect on the cytoskeleton can be explained by its ability to act as a potent GEF.

This assay, as shown in figure 5.9, detects the outcome of an exchange activity (the rise in GTP-Mant loaded G protein) the Mant fluorophore GTP when loaded on the G protein gives rise to the fluorescence intensity that is measured by the spectrophotometer. Thus the explanation for this rise in intensity can be attributed to, as mentioned earlier that FicRL (and not the mutated version) acts a GEF, which produced the high levels of GTP-Mant loaded G protein. This explanation is supported by the mechanism by which GEF function, and also by the fact the two described Fic proteins that target Rho GTPases (VopS and IbpA) both induce PTM (Post Translational

Modification) to GTP-loaded Rac and not GDP-loaded G protein as is the case in this experiment. A likely scenario is that Fic proteins have GEF activity (that required the FIC motif) to exchange GDP for GTP and then cause the PTM (utilizing the fluorophore bound substrate) on the GTP-loaded G protein (figure 5.8).

Another possibility, shown in figure 5.9, is that Fic-RL modifies the G protein (Cdc42 in this case) using substrate carried over in the purification process, and by PTM the Fic protein causes the release of GDP. With the abundance of Mant-GTP in the reaction, the G protein binds to the GTP-Mant substrate and gives the resultant rise in the measured fluorescence intensity. Any PTM on Cdc42 would not be assessed by this experiment, as this experiment does not show what type of PTM is done to the protein, but only reveals that an enzymatic activity has taken place.

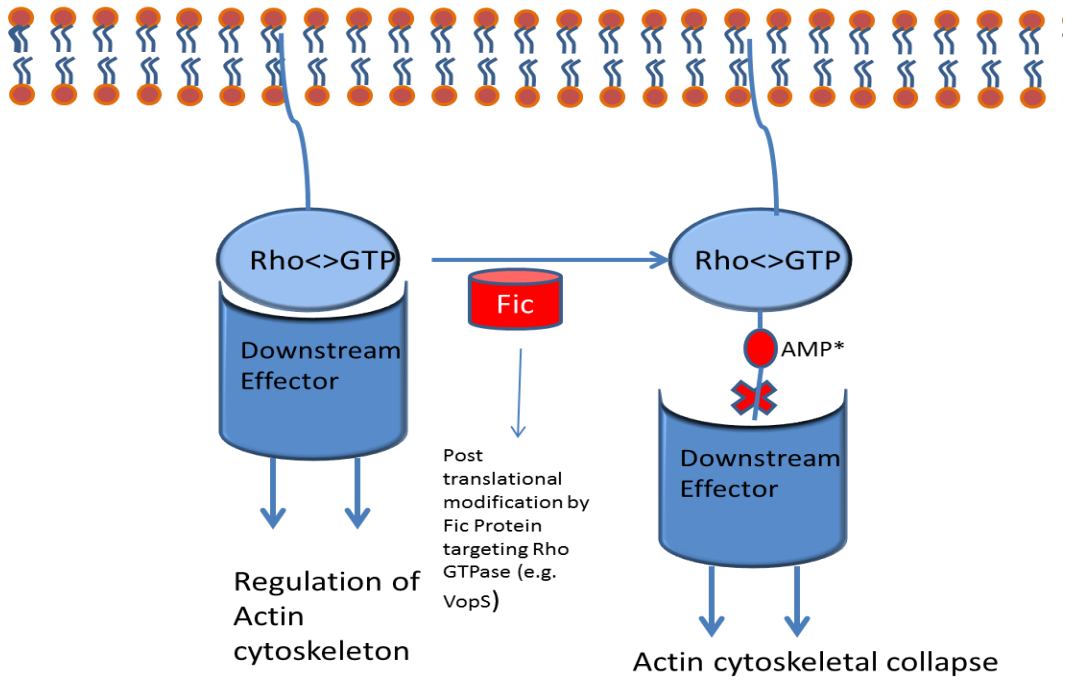


Figure 5-8 depicts the means by which other Fic proteins targeting Rho GTPases, as have been described in the literature. The figure has been modified from the original figure in[41]. Fic proteins target GTP loaded Rho GTPases and modify it by means of PTM causing it to not be able to bind to its downstream effectors. A GEF however

functions by exchanging the GTP bound to the G protein for a GDP and releasing the extra phosphate.

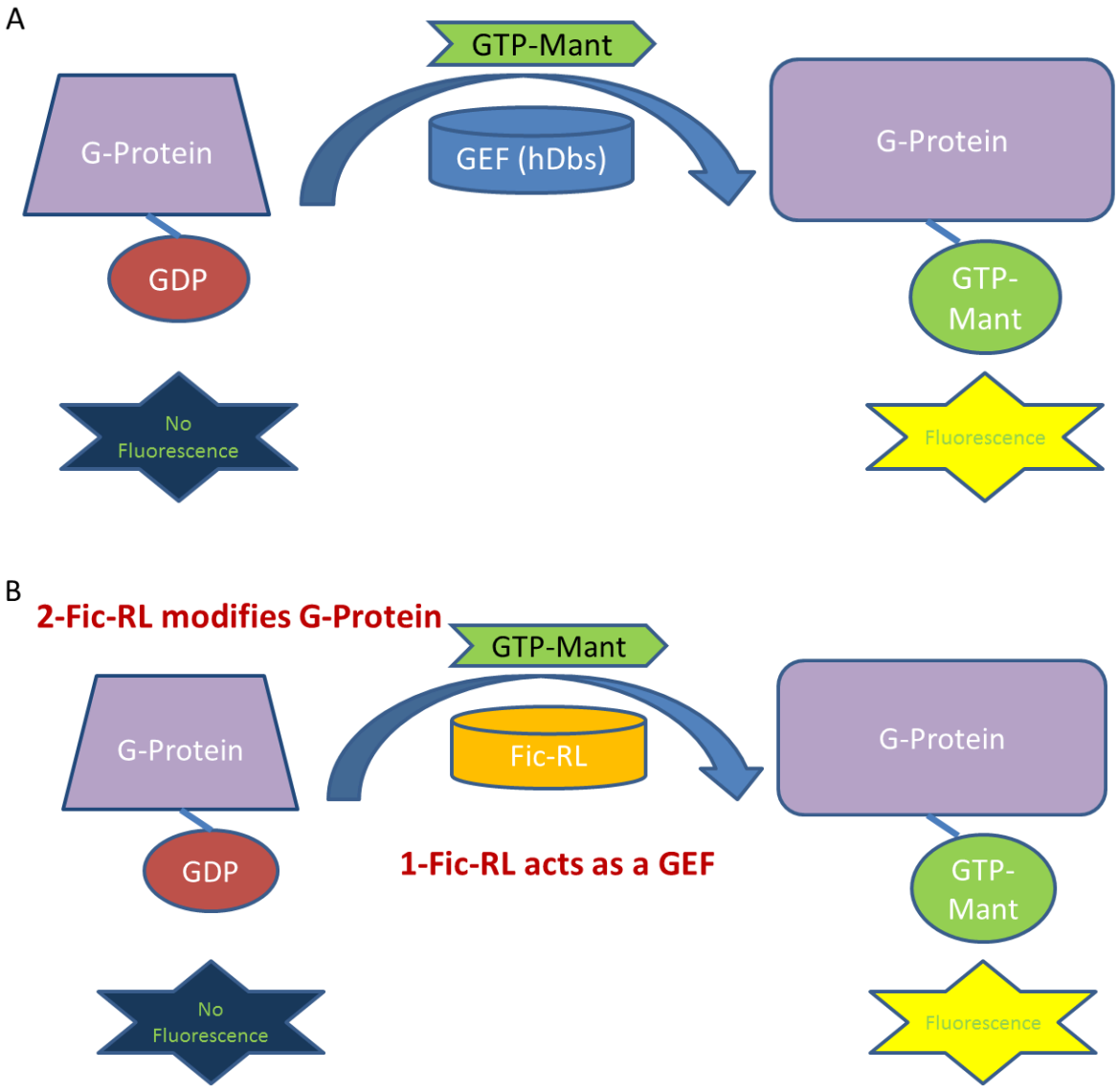


Figure 5-9 the basic mechanism of how the GEF assay works. In a black 96 well plate, fluorescence intensity is measured after all the 3 ingredients for the reaction are added at the same time, namely the GDP-loaded G- Protein (Cdc42 or RhoA or Rac1) the substrate; a fluorophore bound GTP (GTP-Mant) and the GEF to be tested (FicRL, mutant FicRL) or the positive control (hDbs). The GEF catalyses the reaction exchanging the GDP for the GTP which is loaded with Mant fluorophore that is detected only upon its addition to the G-Protein (no fluorescence is detected if no GEF is added or if negative control is added – GST tag alone). In 5.9-B two proposed

mechanisms that could potentially explain the ability of Fic-RL to produce GTP-Mant bound Cdc42. The first mechanism is that Fic-RL acts as a GEF would act similar to what hDbs does in; 5.8-A. The second proposed mechanism is that Fic-RL modifies the GDP-loaded G-Protein (perhaps with substrate carried over from the protein purification step that is inherently bound to Fic-RL e.g. AMP molecule) Fic-RL modifies the G-Protein causing it release the GDP while the abundant substrate in the reaction takes over the place of the GDP (the Mant-GTP).

The results in this chapter do not show exactly what is being added to Cdc42 or if any modification occurs to the Cdc42 that results in the addition to GTP-Mant substrate or the exact mechanisms of any such modification or addition to Cdc42. In future work however the resultant Cdc42 of the exchange assay could be assess by mass spectrometry to reveal what exact modification happened. This study so far however, shows that FicRL exhibits GEF or GEF-like activity to Cdc42 and not to RhoA and Rac1, and mutating the invariant histidine in the Fic motif causes the loss of this function.

FicRL ability to selectively target Cdc42 and hijack its regulatory function by activating it, suggests that *Klebsiella pneumoniae* would benefit from such activation; the hijacking of Cdc42 and consequently of the PAK2 pathway that is activated by Cdc42 would be a mean for an end that would suit that invader's needs [43, 187].
This need could potentially be explained by examining the effector of the target of FicRl (Cdc42), which is group 2 of PAK proteins (p21 activated Kinases). PAK2 are labelled as cellular responses to stress and are activated by Cdc42. PAK2 has the selective role - and not any other member of PAK groups- in promoting cell cytostasis, where Myc protein (an effector of PAK2) is phosphorylated by PAK2 which results in the blocking of Myc protein's ability to bind to DNA, interfering with transcription and the cell's ability to proliferate (cytostasis). Forcing the cell to enter cytostasis would be of great benefit for an invading organism wanting to hide from the host's defences present outside the cell such as the complement system [56, 77]. In turn, this would also explain the role of FicRL in the pathogenesis and why we expect a host with this protein to be more virulent

than an organism without it, (as evident by the reduction of virulence shown in the *Galleria mellonella* infection model). A mutant *K. pneumoniae* lacking the *fic-rl* gene was shown to be less virulent than the wild-type strain), as shown in results chapter 3.

FicRL's ability to produce GTP-loaded Cdc42 and its ability to do so to levels comparable to that positive control (hDbs), gives insight on two possible defining points for its role. Firstly, FicRL is possibly able to recognise the provided GDP-loaded Cdc42. This can be deduced by that fact that it exerted its function selectively on Cdc42 and not Rac1 or RhoA. A precedent in literature is seen in the Fic protein IbpA that also targets rho GTPases, where IbpA crystal structure showed it bound to Cdc42 and was regarded as a model for target recognition of a Fic protein [51]. The same study also shows a structural view of PTM carried out by a Fic protein. This target recognition is followed by binding (GEFs bind to nucleotide bound GTPases before exerting their action), and thus it is possible to conclude that FicRL binds to Cdc42 in order to induce its action assuming it follows the same mechanisms that other similar Fic proteins follow.

Secondly, FicRL, as does the positive control hDbs, was able to catalyse the reaction where first it was able to release GDP from Cdc42. GDP natural dissociation cannot explain the phenomenon observed in this experiment, as the time given for the experiment to run was only 30 minutes, in which comparable levels of GTP Mant-bound G proteins were observed in FicRL to those levels of the positive control hDbs.

GEF bind to their target and cause the release of the bound nucleotide (GDP), the GEF is then released once a new nucleotide is bound to the G protein (GTP). In this experiment the speed at which FicRL was causing the G protein to dissociate its GDP nucleotide hints that this protein might actually function as a GEF. Cdc42 is left to bind to the abundant Mant-GTP substrate and the bond was stable enough to be recorded as it was for the positive control. However, such effects were not observed in the mutant version of FicRL, where Fic-RL which again and eventual release of the now GTP-loaded Cdc42 freeing FicRL to exert function on another Cdc42-GDP complex (this is the recorded intensity that was rising throughout recording and not decreasing), especially that the experiment was optimized to have almost equal amounts of FicRL or hDbs into each reaction.

In this respect FicRL can be described as a proper GEF, whereas other described Fic domain bearing proteins that exert action on the Rho GTPases (such as VopS and IbpA) have been described also as GEF with similar results, although in an indirect fashion [43]. Yarborough et al have also shown that Cdc42 is inhibited when expressing VopS in eukaryotic cell lines and that Cdc42 is unable to bind PAK, its downstream effector (which is expected without the need of experimentation, as it is a known effector of Cdc42). However, they reached the conclusion that VopS acts as a GEF by an indirect in vivo approach, which can be utilised in the future to provide evidence of the effect of any Fic domain bearing protein and the effect of downstream effectors of Cdc42 to link these effects with observed phenotypes.

The experiment performed in this chapter shows that a Fic domain bearing protein of bacterial origin has an ability to act as GEF on a specific target of the Rho GTPases (Cdc42) utilising GTP as a substrate (which is the available substrate usually in the intracellular compartment). However, Yarborough et al show that VopS uses AMP molecule to exert its function. the AMP loaded Cdc42 in their experiment is unable to be released from the complex and thus is locked into an ON state, whereas this work shows GEF function (directly or through PTM utilizing GTP) might not explain if Cdc42 would also be locked in an ON state as well (however the immunofluorescence work suggests that it does as both VopS and FicRL have similar effects on the cytoskeleton after transfection).

The in vitro exchange assay done in this phase does not necessarily show the in vivo function of FicRL, nor the substrate that FicRL might use in vivo. FicRL might not be very selective in its use of substrate if it does indeed use another substrate in vivo to turn Cdc42 into an always-ON state as does VopS (which is again explained by the fact that VopS and FicRL both show similar effects on the cytoskeleton of the eukaryotic cell they are transfected to).

One might conclude that VopS might also use GTP as a substrate in an in vitro model where GTP is the only provided substrate but uses AMP in vivo for a substantial gain in escaping host defence (AMP addition locks the Cdc42 in its ON state but the addition of GTP is reversible) however this has to be shown experimentally either by testing VopS with an exchange assay or testing FicRL with an in vivo model as was VopS.

The lack of other fluorophore loaded substrates hindered our search to test whether FicRL is able to utilise other substrates and add them directly to Cdc42, and this remains to be part of future endeavours.

It is noteworthy that the mutation of the hydrophilic Histidine in the conserved region to the hydrophobic Alanine caused a possible difference in the solubility (as each protein was retrieved at higher concentrations at two different temperatures). This can be attributed to different folding or misfolding that are usually caused by conformational changes within the protein that results in polarity shifts and resultant exposure (or hiding) of polar regions with an end result of different interaction in polarity with water. It is however, unlikely that this is the cause for the loss of GEF effect seen in the wild type vs mutant forms of FicRL, where the mutant form was not able to exchange GTP for GDP in any of the 3 Rho GTPases tested including Cdc42. The likelier hypothesis, supported by evidence in the VopS study, is that the loss of function of the mutant FicRL was due to the loss of function exerted by the conserved histidine that was removed in the mutant form of FicRL.

Cdc42 role in cancer formation reported in scientific and medical literature raises the question of cancer formation and Fic proteins and a possible link to tumour growth or promotion (or suppression[188]) is put forth[187]. The link has been previously made between bacterial invasion and cancer development [189]; however, it is only a speculation at this point linking the effect of Fic domain bearing proteins and their mechanism of inactivation or activation of Cdc42 and resulting effects on the cytoskeleton and apoptosis with development or maintenance of any tumours. The link could be the subject of future research.

5.5 Final Discussion of Results Chapters 3, 4 and 5, and Future Plan

This project was aimed at identifying the role of a specific domain that was found on a genomic island characterized in *K. pneumoniae*, namely KpGI-2. The protein coded by the gene was bearing a Fic domain, further analysis utilizing bioinformatics tools has revealed the presence of 5 homologs of protein bearing Fic domain in *K. pneumoniae*.

The significance of these Fic protein on the biology of *K. pneumoniae* was revealed upon further analysis utilizing in-vivo and in-vitro analysis of the effect of these proteins.

Firstly, of the five fic proteins, one such protein was shown to be conserved in 100% of isolates of *K. pneumoniae* as shown in the 80+ fully sequenced *K. pneumoniae* strains present on the NCBI database, namely the Fic-RL protein. Determining if any of these five Fic proteins were secreted has revealed that only the Fic-RL protein was present in the secretory fraction of proteins collected when strains of *K. pneumoniae* were transformed with a vector that carried a coding sequence for the Fic-RL protein tagged with a FLAG tag. By using temperature shift, the strain was inducing to secrete proteins, which were collected and purified and the presence of Fic-RL was observed. Furthermore, by utilizing different *K. pneumonia* mutant strains, that lacked various types of T4SS or T6SS, it was revealed that the strains lacking the T4SS present on ICE1 conjugative element, has lost the ability to secrete Fic-RL. This finding was in conformance with studies found in literature, where in other bacteria, Fic proteins were secreted by T4SS or T3SS, and the effector Fic protein were carrying out toxic type function on the target cells.

In-vivo analysis using a killing assay, utilizing the wax moth, *Galleria mellonella*, has revealed that the deletion of *fic-rl* gene has caused a significant attenuation of the strain that lacked this gene in strain HS04160. This was later confirmed in another killing assay that compared attenuation levels when ICE1 was deleted from strain HS11286 Versus a

deletion of *fic-rl* gene in the same strain. It was noticed that both deletions caused a very comparable and significant levels of attenuation in this bacterium.

The impact of this T4SS effector Fic-RL, on the effectiveness of *K. pneumoniae* to cause infection in this killing assay prompted in-vitro analysis to observe the effect of this protein on living cells in a culture environment.

Fic proteins in other bacteria were observed to be utilized to disrupt the actin cytoskeleton of the target cell, this disruption was caused by causing PTM on regulatory element of the cytoskeleton, which is controlled by small GTPase families such as Rho and RAB GTPases, which would either cause direct cytotoxicity to the cell (when affecting Rho GTPases) or prevent the destruction and escape the cell defenses (when affecting Rab GTPases). Transfection of vectors carrying one of each of the five Fic protein revealed in this study in *K. pneumoniae*, has revealed that Fic-RL has caused direct cytotoxicity to human kidney cells (HK-2) and human alveolar cells (A-549), as observed by cytoskeletal collapse (cell rounding) or membrane ruffling of these cells under confocal or fluorescence microscopy. This effect was not observed in other Fic proteins, nor when a mutant version of Fic-RL was expressed in these cells (the mutant had the conserved Histidine in the Fic motif replaced with an Alanine, which was shown in other studies to be responsible for the PTM function and ultimately disrupt the Fic protein's function).

To understand the effect observed on the cytoskeleton, an enzymatic analysis was performed. The analysis utilized purified proteins of the Rho GTPase family (CDC42, RhoA and Rac1) and measured the fluorescence when a protein that modified any of these proteins was introduced, the assay also added the fluorophore bound substrate the reaction would use to measure fluorescence. The assay has revealed that adding Fic-RL to Cdc42 (and not RhoA or Rac1) has caused a significant increase in fluorescent that was very close to the fluorescence observed when a positive control (a known GEF HDbs protein) was added to Cdc42. The mutant form of Fic-RL was unable to cause any increase in fluorescence.

This assay has revealed that the target of the Fic-RL protein could potentially be Cdc42, and by form of a PTM, the effect seen under the confocal microscopy on the human cells is can be explained by a PTM of Fic-RL on Cdc42, which is conformant with published data on other Fic proteins in other bacteria (such as VopS and IbpA).

As of April 2017, as the author, and to the best of my knowledge, this was not shown in any other data published or otherwise. The significance of the results shown in this project, reveal the previously unknown role of Fic-RL in the ability of *K. pneumoniae* to cause infection and induce cytotoxicity to human cells. The understanding of which can help combat this dangerous bacterium, especially that a similar protein (IbpA), when was vaccinated against IbpA has protected cattle from developing pneumonia. A similar approach may be developed to produce a vaccine that can protect hosts from developing *K. pneumoniae* infections, which will have a significant impact on human health, especially with the rise of hypervirulent and antibiotic resistant strains that leave clinicians with little to no options to fight against.

Future work will be focused on two approaches, first is to further understand the enzymatic mechanism of Fic-RL. by analysis Cdc42 that has been modified by Fic-RL and pinpoint what change (and where) was made by Fic-RL. Secondly is to test the ability of Cdc42 modified by Fic-RL protein to bind to its downstream effector (such as PAK). Secondly, further work will focus on clinical effects of developing a vaccine against this protein, first by utilizing animal models and observing whether a vaccine produced against this protein would protect these animals from developing infections of K. pneumoniae, and whether this approach is feasible in humans.

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