

OmpK36-mediated carbapenem resistance in *Klebsiella pneumoniae*

PhD Thesis

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partial fulfilment of the requirements for the degree of Doctor of Philosophy

Supervisors: Professor Gad Frankel,
Dr Abigail Clements and Professor Stephen Brett

Joshua Liang Chao Wong

MRC CMBI, Imperial College London

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Candidate's statement of originality

Excepting the contributions acknowledged below or referenced in-text, I hereby confirm that this thesis constitutes my own work.

Figure 4.3. The X-ray crystallography and analysis was carried out by Dr Hok Sau Kwong and Dr Maria Romano in the laboratory of Dr Kostantinos Beis (Imperial College London).

Figure 4.4 and 4.5. Liposomal swelling assays were carried out by Dr Hok Sau Kwong and Dr Maria Romano in the laboratory of Dr Kostantinos Beis.

Figure 5.1 and 5.2. The ST258/512 phylogeny and homosplasia analysis was carried out with Dr Sophia David in the laboratory of Professor David Aanensen (Big Data Institute, University of Oxford)

Figure 5.4 and 5.5. The DMS-MaPseq was carried out by Mr Jia Woo in the laboratory of Dr Silvi Rouskin (Harvard Medical School, Harvard University).

Abstract

Klebsiella pneumoniae (KP) is a common nosocomial pathogen that is frequently responsible for pneumonia, urinary tract infections and bacteraemia in patients with long term health challenges. Such infections can progress to sepsis, often necessitating intensive care support. Antibiotics are the cornerstone of treatment, however, mounting resistance to a current last-line class, the carbapenems, threatens to render some infections untreatable. Robust carbapenem resistance is achieved by two divergent but synergistic mechanisms in high-risk, globally disseminated, resistant sequence types (ST), such as KP ST258. Firstly, plasmid-borne carbapenemase enzymes hydrolytically inactivate carbapenems in the bacterial periplasm. This is complemented by chromosomal mutations in two outer membrane (OM) porin genes, *ompK35* and *ompK36*, that reduce drug diffusion into the periplasm. Whilst truncations in OmpK35 are well described widespread phenomena, loss of OmpK36 is rarely observed in clinical isolates. In this thesis I focus on identifying and characterising the precise molecular mechanisms by which mutations in the dominant KP porin, OmpK36, impair carbapenem diffusion. I start by focusing on OmpK36 pore-constriction and identify a di-amino acid Glycine-Aspartate insertion in the pore diameter-determining motif, loop 3. This structural modification reduces carbapenem diffusion and increases the minimum inhibitory concentration. When the fitness cost of OmpK36 pore-constriction is tested, in a novel murine ventilator-associated pneumonia model, I identify a fitness disadvantage only when tested in competition with wild-type strains. This contrasts with a recurrently emerging novel *ompK36* mutation, a 25c>t, identified in a large KP ST258 genome collection. The 25c>t depletes OmpK36 from the OM by inducing an inhibitory mRNA secondary structure and leads to significant attenuation *in vivo*. Finally, bringing this together, when meropenem treatment is instituted in this translational model, both mutations are demonstrated to be advantageous to KP and result in treatment failure, whereas strains expressing wild-type OmpK36 are susceptible to therapy.

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I think it is traditional here to thank work colleagues followed by friends and family. I am going to have to break with tradition as so many of those I have worked with in Gadi’s lab have become friends and, therefore, there is no distinction. Without the help and guidance of my three supervisors (Gadi, Abi and Steve) none of this would not have been possible. It was always going to be a challenge to start a new project from scratch, on a new pathogen and in a new area of study- much of this has relied on teamwork and being afforded the freedom to explore new ideas. The combination of strong scientific (Gadi and Abi) and clinical (Steve) supervision has been key in making this project a success. This is especially true as this work represents Imperial College London’s first severe bacterial infection model.

There are a few colleagues that merit specific mention. Firstly, Wen Wen Low, my first masters student and current PhD student who took everything I taught her and now does it better and quicker! Louise Kerry, a founding member of team *Klebsiella*, whose organisational skills kept me in check and ensured that the stuff I needed to do experiments with was actually available. Julia Sanchez-Garrido helped me turn dreams into a reality and add multiple analytical variables to our animal model, especially with respect to host responses. Whilst this represents the core *Klebsiella* team, we are expanding and have new members including Jonny Bradshaw, who has already made fundamental steps in strengthening our work. Dani Carson, I am very sorry that I made you do night shifts in the animal facility and that we never got that work published! Those not mentioned by name who make my life great or make me want to come to work everyday know who they are.

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Abbreviations

μl microlitre ml millilitre L litre 5'UTR 5' untranslated region A aa aminoacid a Adenine A Alanine ALT Alanine transaminase ANOVA Analysis of variance Arg Arginine ARDS Acute Respiratory Distress Syndrome Asp Aspartate ASPA Animals (Scientific Procedures) Act 1986 AST Aspartate transaminase ATCC American Type Culture Collection AVI Avibactam AWERB Animal Welfare Ethical Review Body B BALF Bronchoalveolar Fluid BAM β -barrel assembly machine C c Cytosine CC Cross Collaborative CD Cluster of Differentiation CFU Colony Forming Units CRKP Carbapenem resistant <i>Klebsiella pneumoniae</i> CT Computed tomography CXCL-1 (C-X-C motif) ligand 1 D D Aspartate DPBS Dulbecco's Phosphate Buffered Saline E EDTA Ethylenediaminetetraacetic acid Ent Enterobactin ERT Ertapenem ESBL Extended spectrum β-lactamase F FBS Fetal Bovine Serum G g gram GA Genetically Altered GD Glycine-Aspartate GFP Green Fluorescent Protein Gm Gentamicin	H h hour hpi hours post infection HAP Hospital-acquired pneumonia HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid HES Hospital Episode Statistics HV Hypervirulent I i.p. intraperitoneal ICU Intensive Care Unit IFNγ Interferon Gamma IL-6 Interleukin-6 IN Intranasal IPM Imipenem IS Insertion Sequence IT Intratracheal K kg kilograms Kn Kanamycin KP <i>Klebsiella pneumoniae</i> KPC <i>Klebsiella pneumoniae</i> carbapenemase L L3 loop 3 L4 loop 4 LB Luria Broth Leu leucine LPS Lipopolysaccharide LSP Leucine-Serine-Proline Lys Lysine M M9 M9 minimal media Mbp Mega basepairs MEM Meropenem mg milligrams MLST Multilocus Sequence Type MIC Minimum inhibitory concentration N NDM New Delhi Metallo beta-lactamase NHS National Health Service
--	--

Abbreviations

O

OA Oropharyngeal Aspiration
OD Optical Density
OM Outer membrane
ORF Open reading frame
OXA Oxacillinase

P

PBS Phosphate Buffered Saline
PCR Polymerase Chain Reaction
PICO Population, Intervention, Comparison, Outcomes
Pro Proline

R

R Arginine
REL Relebactam
RFP Red fluorescent protein
Rif Rifampicin
rmsd root mean square deviation

S

s.c. subcutaneous
SDM Site Directed Mutagenesis
SDS Shine-Dalgarno Sequence
SDS-PAGE Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
Ser Serine
Sm Streptomycin
SS Signal Sequence
ST Sequence Type

T

t Thymine
TD Threonine-Aspartate
TNF Tumour Necrosis Factor
TLR Toll-like Receptor

U

u Uracil

V

VAB Vaborbactam
VAP Ventilator-acquired pneumonia

W

WHO World Health Organisation
WT Wild-type

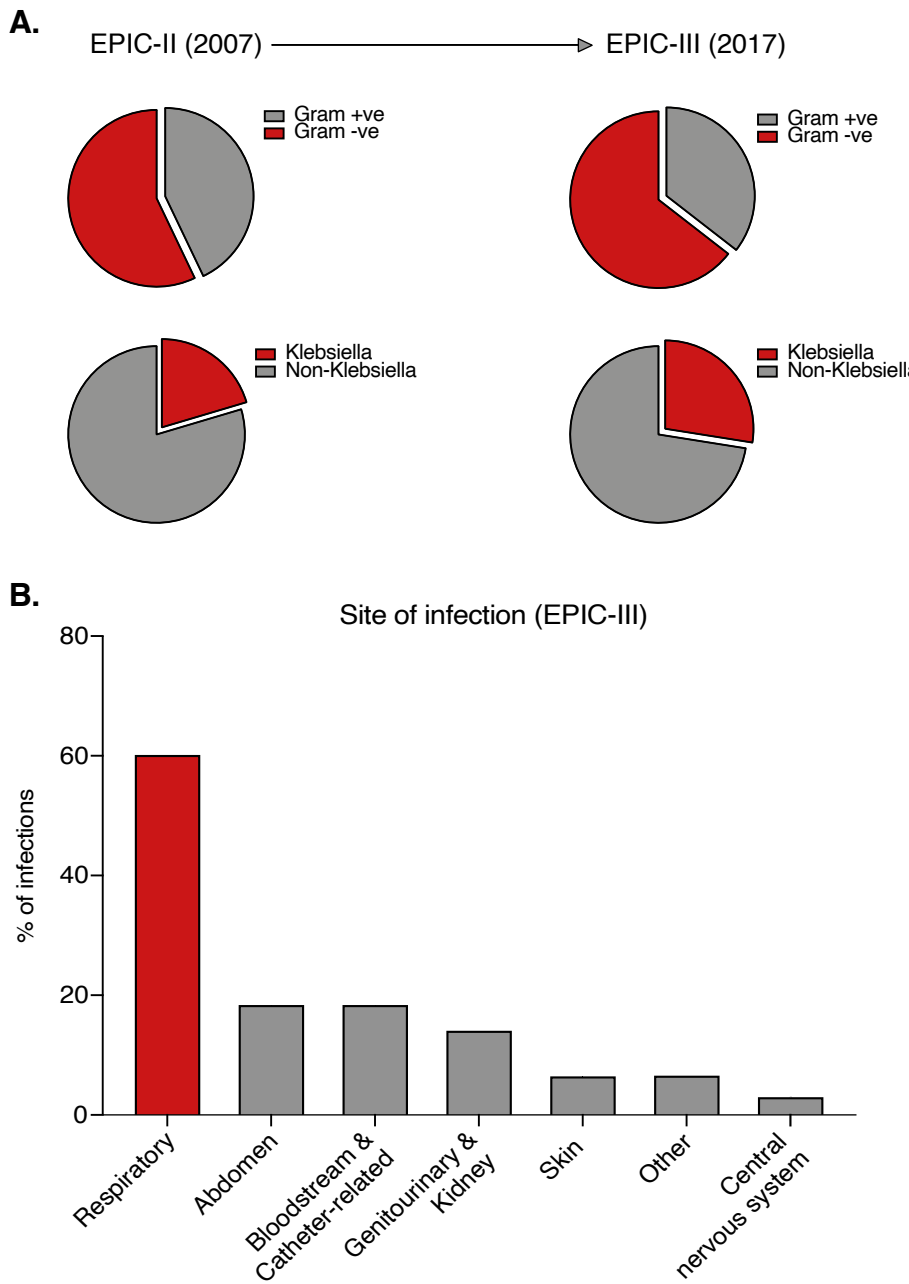
1. Introduction

1.1 *Klebsiella pneumoniae*

Klebsiella pneumoniae (KP) was first described as the causative agent in a fatal necrotising pneumonia by Friedlander in 1882¹. It is now classified as a WHO critical priority organism and urgent threat to global health, due to alarming levels of resistance to last line antibiotics². It typifies a successful modern pathogen as it thrives in the nosocomial niche, frequently colonising inpatients and causing invasive infection at multiple body sites. Classical KP isolates most commonly cause pneumonia and urinary tract infections often progressing to bacteraemia³. The severe systemic clinical manifestation of such infections are the syndromes of sepsis and septic shock⁴. Here, the host mounts an excessive, dysregulated and ultimately injurious response to infection which results in up to one in three to one in six patients dying^{5,6}. Life-threatening organ dysfunction requires levels of support that can only be provided in the specialised resource rich critical care environment.

KP infections generally occur in patients with health problems who are hospitalised as a result of complications of their established conditions. Medicine has made significant inroads in reducing mortality due to bacterial infections⁷; however, this improvement is predicated on our ability to deliver prompt and effective antibiotic chemotherapy⁸. The prevalence of KP as the causative agent of human disease, in combination with its predilection towards acquiring antibiotic resistant traits, threatens to jeopardise the fundamental improvements that have been made in reducing the mortality from severe infection. This is compounded by the absence of a licensed vaccine or alternative non-antibiotic treatment options, such as therapeutic monoclonal antibodies for KP. Whilst classical strains are an established pervasive threat, hypervirulent strains (HV) have now emerged in Southeast Asia and are spreading globally⁹. Their pathogenesis is distinct from classical KP infection. The development of liver abscesses is characteristic and these strains breach host defences with ease via the gastrointestinal tract. Metastatic infection to extra-hepatic sites is common with spread to soft tissues (necrotising fasciitis), the eyes (endophthalmitis) and the central nervous system (meningitis) frequently reported⁹. In contrast to classical strains, HV strains can cause infections in healthy individuals. This shift in pathogenesis results in community acquisition of invasive infection. Several virulence factors are associated with a HV phenotype, such as excessive capsule production, hypermucoviscosity and the acquisition of multiple siderophore systems. However, the contribution of individual factors mediating increased invasiveness in humans remains unknown⁹. A very real threat, already reported in the literature¹⁰, is the convergence of classical and HV lineages, resulting in highly pathogenic strains rendered untreatable by broad spectrum antimicrobials.

Figure 1.1 Infection and *Klebsiella pneumoniae* in Intensive Care



Data obtained from the EPIC-II and EPIC-III international point prevalence studies of infection in global Intensive Care Units^{12,13}.

A. In the ten years between data collections the proportion of Gram-negative infections has increased (from 57.1% to 64.5%). The proportion of Gram-negative infections caused by KP also increased in the time period (from 20.4% to 27.5% of all isolated Gram-negative organisms) indicating a large increase in the burden of critical care KP infections.

B. The sites of primary infection in critical care patients from the EPIC-III study. 60.1% of all infections were proposed to have an origin in the respiratory tract.

1.2.1. *Klebsiella pneumoniae* sepsis in Intensive Care

Intensive Care Units (ICUs) have been coined the 'epicentres of infections'¹¹. Bacterial infection resulting in sepsis is extremely common in this environment, either as cause or consequence of admission; in a large international point prevalence ICU study (EPIC-II, 2007), 51% of patients were considered infected and 71% were receiving antibiotics¹². When this study was repeated 10 years later (EPIC-III, n=15,202 patients, 2017¹³), 53.5% of patients were considered infected. Of note, in the 10-year period between these studies the proportion of Gram-negative infections appeared to have increased from 57.1% to 64.5% of all cultured isolates (**Figure 1.1A**). The proportion of these Gram-negative infections caused by KP also

increased, from 20.4% to 27.5% of the total (**Figure 1.1A**). This data indicates a net increase of KP infections in the sickest population of hospital inpatients. In this environment the host-pathogen interaction shifts in the favour of pathogens. This shift is multifactorial and includes immunosuppression from concomitant disease processes and the requirement for multiple, invasive interventions that overcome normal protective barriers. It is also known that the local ICU environment is enriched with pathogenic bacteria¹⁴. This is further compounded by severe illness and antibiotic administration that both perturb the host microbiota¹⁵. The resultant dysregulation leads to the expansion of pathobionts, such as KP, at multiple body sites and mucosal surfaces¹⁶.

Critical care is often associated with immunosuppression, however, the exact mechanisms underpinning this remain poorly described. This has been attributed to the heterogeneity in the patient population and mechanisms are likely patient and co-morbidity specific. In EPIC-III many co-morbidities were independently associated with infection including the presence of lung disease (chronic obstructive pulmonary disease), cancer, diabetes, chronic kidney failure, HIV infection and immunosuppression¹³. Whilst in the final two host immune function is clearly compromised, all the other conditions are associated with infection and thus, immune defects are likely. Perhaps the strongest data supporting the notion that critical care is associated with immunosuppression is the higher risk of infection with increasing length of ICU stay. Whilst this may be confounded by a higher exposure to environmental pathogens the chances of infection steeply increase following 2 days of admission, peaking in the 8 to 14 day post admission period (odds ratio of infection 4.69 (95% confidence interval 3.71-4.77), $P < 0.001$)¹³.

The majority of infections in the ICU population have the lungs as their primary focus. In the clinical study, EPIC-III, 60.1% of patients had infections that originated in the respiratory tract (**Figure 1.1B**)¹². Hospital-acquired pneumonia (HAP, defined as pneumonia acquired >48 hours post hospital admission) and ventilator-acquired pneumonia (VAP, defined as pneumonia acquired >48 hours of artificial mechanical ventilation) are the major subtypes of respiratory infections occurring in the critically unwell. These are distinct clinical entities with different bacterial aetiologies from community-acquired pneumonia¹⁷. In culture-positive HAP there is shift in spectrum towards Gram-negative and multidrug resistant organisms¹⁸. VAP is a distinct subtype of HAP as it occurs only in patients undergoing mechanical ventilation in the ICU setting. Despite the huge burden of lung infections in critical care there is a paucity of data explaining the mode by which KP infection reaches the lungs of patients. Theories have included direct aspiration into the lower respiratory tract, however, simultaneous nasopharyngeal KP carriage and invasive lung infection do not appear to directly correlate excluding this as a direct cause³.

1.2.2. Antibiotic resistance in Intensive Care

ICUs use large quantities of antibiotics. Patients present with life threatening infections and the prompt administration of effective antibiotics has a clear survival benefit⁸. This presents a dilemma for treating physicians. At the time of presentation, the results of samples cultured to identify the causative organism are not readily available and broad-spectrum antimicrobials are frequently used, often in combination, as first line agents. It is hypothesised that their administration has driven the selection and expansion of antibiotic-resistant bacteria in this environment¹⁸. On a patient-by-patient basis, where delays in effective antibiotics are implicated in an increased odds of death (11% per hour of antibiotic delay in one study⁸), it is difficult to justify the use of a narrow-spectrum agent that may not be effective. This putative survival benefit has led to the increased administration of the very broad-spectrum agents, such as the carbapenems. Several novel approaches to the real-time detection of causative agents and their associated antibiotic resistance have thus far failed translation from the bench into clinical practice or lacked effectiveness for implementation reasons¹⁹.

1.3. Genetic heterogeneity in KP

There is significant genetic heterogeneity in KP isolates. In the first large sequencing study comprising 328 whole genome sequences, including clinical and environmental isolates, the median KP genome size was 5.7 Mbp, with each genome encoding a median of 5,705 genes²⁰. Only a small fraction of genes (1,743) could be defined as common or 'core' and present in greater than 95% of isolates. The remaining genes, representing a median of 3,817 per genome, were termed 'accessory' as they were present in less than 95% of genomes. It is hypothesised that this genetic diversification underlies this pathogen's species-level success, however the components of the 'accessory' genome driving this are unknown. This high level of intraspecies variability poses a significant challenge in the scientific study of KP, as strain specific biology is significant, due to broad genetic divergence.

Despite this genetic variability several schemas have been developed to classify strains into tractable lineages. The most broadly utilised is multilocus sequence typing (MLST)²¹. MLST classifies strains according to a unique combination of 7 genetic loci and 3453 sequence types (STs) are currently defined. Whilst this was initially based on a Sanger-sequencing, low throughput approach, MLST analysis is now built into multiple new platforms that can rapidly assign ST from next generation sequencing data²². Such advances in the field, which have occurred over the period during which the work in this thesis was conducted, have allowed me to rapidly analyse large datasets and make use of the increased availability of public KP genomes. The rapid and global expansion of the ST258 KP since 2001 is one example of a high-risk global ST²³. ST258 strains have expanded intimately the expression of the KPC carbapenemase

enzymes which confer resistance by carbapenem hydrolysis. In the large prospective KP study, EuSCAPE, encompassing whole genome sequences from 1,717 KP strains across Europe, ST258 (and its single locus variant ST512) were predominant²⁴. This study was published in 2019 and collected isolates between November 2013 and May 2014. Together with 3 other STs (ST11, ST15, and ST101) they comprised 69.9% of the carbapenem non-susceptible KP in the collection. The biology that has favoured the success of these STs in mediating the majority of carbapenem resistant KP disease remains unknown.

1.4.1. Virulence Factors in *Klebsiella pneumoniae*

KP is a virulent human pathogen that lacks an armamentarium of offensive strategies. Instead, its success appears to lie in the evasion of host responses³. The major virulence factors in KP have been identified by transposon mutagenesis screens and tested in murine pneumonia models^{25,26}. These screens consistently highlight the importance of three virulence factors- capsule, lipopolysaccharide (LPS) and siderophores. Most work has focussed on dissecting the function of individual virulence factors in a reductionist approach, however, the interplay between them, and how they co-operate together *in vivo* remains elusive. Whilst other virulence factors have been explored in KP, such as type I and type III pili, which promote adhesion, their role may be restricted to the host site of infection, such as observed in KP urinary tract infection colonisation and persistence²⁷.

1.4.2. Capsule

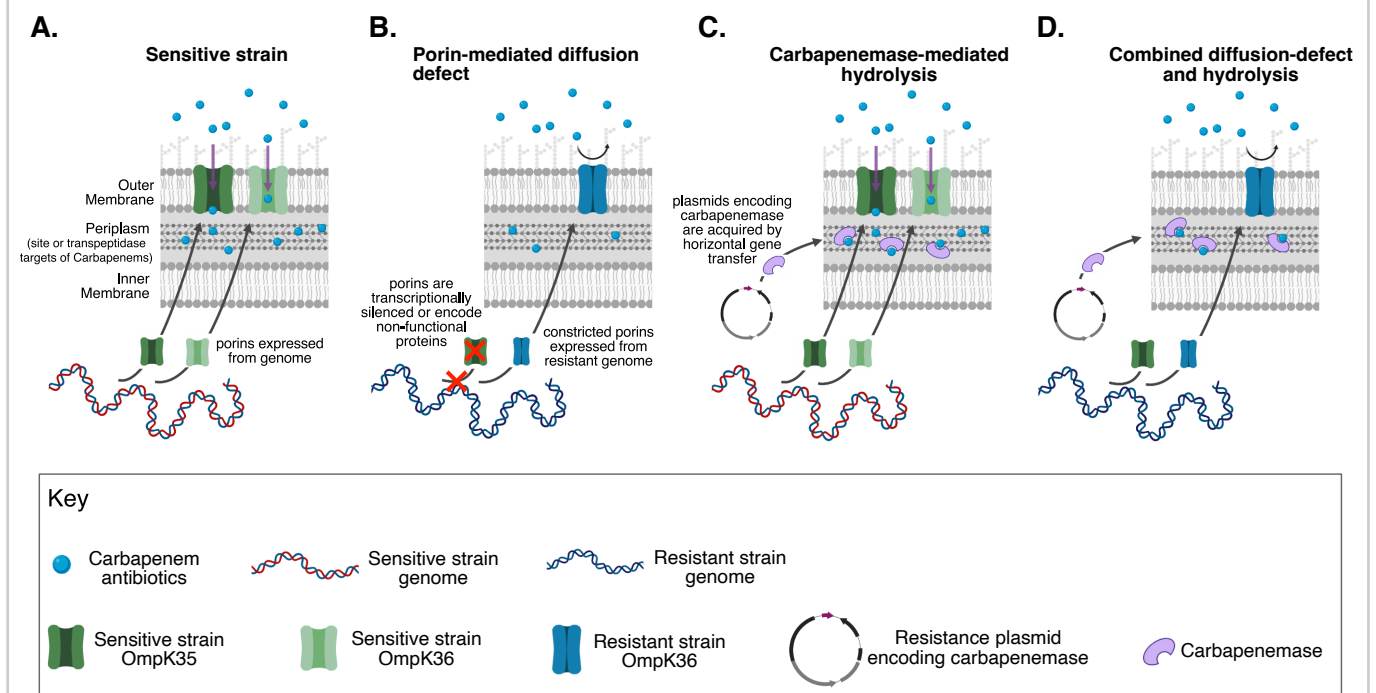
The capsule is a key virulence factor in both classical and HV KP. The *cps* (capsule synthesis and export) locus is chromosomally encoded and is conserved in regional organisation across the KP phylogeny²⁸. K1-K78 capsule types were defined by classical serotyping, which has now been superseded by *wzi*-genotyping²⁸. Acapsular mutants are avirulent in murine pneumonia models²⁹. However, these mutations were constructed *in vitro* and the proportion of circulating clinical isolates, lacking capsule, is not reported in the literature. In KP transposon mutagenesis libraries, strains demonstrating the highest attenuation *in vivo* have disruptions within the *wzi* gene, presumably due to its key role in capsule assembly³⁰. The *rfaH* gene, which encodes a transcriptional anti-terminator of long virulence operons, such as the *cps* gene cluster, was found to contain the second highest number of insertional disruptions in attenuated strains. This highlights KP's reliance on capsule to maintain pathogenic potential²⁶ with further evidence provided by the presence of plasmid or chromosomally encoded *rmpA-C* locus in HV KP strains. This locus comprises *rmpA*, which is a positive regulator of both *rmpC* and *rmpD* expression. These latter gene products drive the hyperencapsulation and hypermucoviscosity, respectively, and their deletion results in attenuation in murine models of KP infection^{31,32}. Capsule mutants demonstrate a deficiency in serum

resistance and enhanced opsonophagocytic-mediated killing, which may underlie and contribute to the observed *in vivo* fitness defect³³.

1.4.3. Lipopolysaccharide

LPS O-antigens demonstrate less variation than capsule K-antigens in KP. Nine O-antigen types could be identified in one series of 638 clinical isolates³⁴. 80% of isolates tested belonged to the O-antigen serogroups O1, O2, O3 and O5. *In vitro* engineered short chain (rough) mutants are more sensitive to serum killing and this has been attributed to an inability to inhibit antibody-mediated C1q binding and complement-cascade activation³⁵. KP further evades immune recognition, by masking the surface

Figure 1.2 Carbapenem-resistance is achieved by a combination of two synergistic mechanisms in KP



- A.** Sensitive (wild-type strains) express two outer-membrane porins, OmpK35 and OmpK36, which play important physiological roles in the allowing the diffusion of hydrophilic molecules across the impermeable outer membrane (OM).
- B.** Porin mutation, either by loss-of-function (deletion or truncation) or expression of mutants with altered diffusion properties, occurs in the high risk clonal, antibiotic resistant population. These changes alter the OM permeability to drugs, however the effect of these mutations on the diffusion of nutrients and, therefore, KP fitness, is unknown.
- C.** Carbapenemase genes are acquired on large resistance plasmids by conjugal transfer. These enzymes hydrolytically cleave and deactivate carbapenems in the periplasm.
- D.** A combination of porin mutation and carbapenemase expression result in robust high levels of resistance in circulating carbapenem-resistant strains.

epitopes in a capsule- and LPS-dependent fashion. Some O-types (e.g. O1) appear to do this in a more efficient manner, which may be due to their higher molecular weight O-antigen chain^{35,36}.

1.4.4. Siderophores

The host response to infection sequesters free iron which limits bacterial growth. Mammalian acute phase reactants (e.g. transferrin) bind free iron with high affinity which in turn reduces bioavailability. The core genome of KP encodes the siderophore enterobactin (Ent) to facilitate iron uptake²⁰. Ent binds Fe³⁺ ions with high affinity and is taken up by KP in a *fepA/tonB*-dependent manner. Mammalian hosts counteract this process by upregulating Lipocalin-2 (LCN-2) production, which binds to and neutralizes Ent. Clinical KP pneumonia isolates encode additional siderophores and associated uptake systems (Aerobactin and *iutA*, Salmochelins and *iroN*, Yersiniabactin and *ybtQ*)³⁷. These alternative siderophores can evade LCN-2-mediated inhibition of Ent. In keeping with this, in murine pneumonia models, the sequential deletion of LCN-2 resistant siderophores limits KP lung CFU counts and dissemination into the blood, liver and spleen³⁸.

1.5.1. Carbapenem resistance in *Klebsiella pneumoniae*

Resistance to antimicrobials in the ESKAPE (*Enterococcus*, *Staphylococcus aureus*, *KP*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter spp.*) pathogens are considered the most important antibiotic resistant threats³⁹. This group accounts for the majority of hospital and healthcare-associated antimicrobial resistant infection. Carbapenems are an important class of antibiotics used in the treatment of life-threatening infections³⁹. The acquired resistance to these agents by KP and other Gram-negative organisms is an increasing global problem that potentially renders this class ineffective. In a US study, covering long-term facilities between 2014 and 2015, almost 25% of KP isolates were carbapenem-resistant (CRKP)⁴⁰. In some countries, such as Italy and Turkey, CRKP strains are now endemic⁴¹. Hospital-acquired CRKP infection has high mortality and there may be an increased risk of death when infection is caused by carbapenem resistant versus carbapenem sensitive strains (42% vs 21%)⁴². Importantly, while this may be due in part to the potential inferiority and known toxicity of the few alternative antibiotics, it illustrates the success of KP as a key antibiotic resistant pathogen and our reliance on this class of antibiotics. Estimates based on the European Centre for Disease control data suggested that CRKP in Europe resulted in 15,947 infections with 2,115 attributable deaths in 2015 alone⁴³. The economic burden of antibiotic resistant KP was further explored and CRKP together with extended spectrum β -lactamase (ESBL)-producing KP infections comprised 25% of the total disability-adjusted life years lost to all multi-drug resistant infection⁴³.

The expression of carbapenemases, in tandem with the limitation of antibiotic influx through the outer membrane (OM) by porin modifications, act together to generate a robust carbapenem resistant phenotype (**Figure 1.2**). These two mechanisms act in concert to effectively lower carbapenem

concentrations at the site of their transpeptidase targets in the periplasm and abrogate the bacteriocidal effect of these drugs. Whilst multidrug efflux pumps, such as those encoded by the *acrRAB* operon are present in KP, mutations in this system have not been evaluated in multidrug resistant STs. *acrB* KP mutants have been constructed *in vitro* and demonstrate increased susceptibility to a broad spectrum of antimicrobial agents, but enhanced functioning of the system has not been demonstrated as an adaptive trait in drug resistant KP clones⁴⁴.

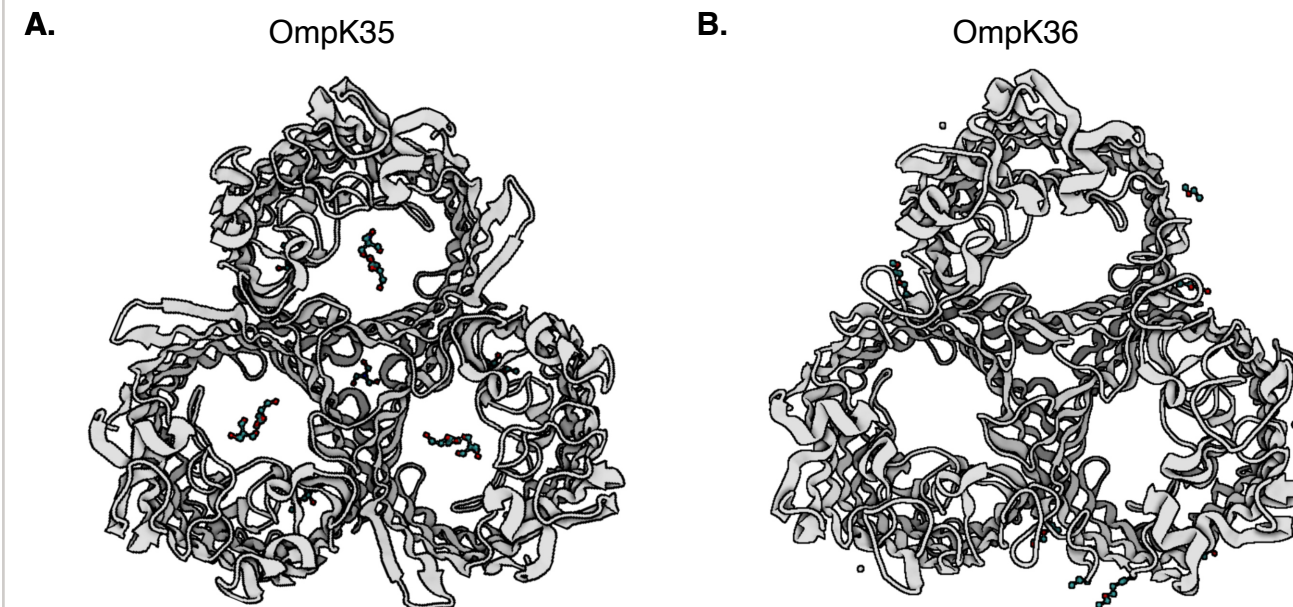
1.5.2. Carbapenemases in *Klebsiella pneumoniae*

Carbapenemase enzymes are usually encoded on large conjugal resistance plasmids^{45,46} and inactivate carbapenems by hydrolysis (**Figure 1.2C&D**). The KPC carbapenemase enzymes are most encountered in CRKP. These enzymes were first described in an isolate from North Carolina in 2001⁴⁷. They are Ambler Class A serine carbapenemases with broad hydrolytic activity against carbapenems, cephalosporins and penicillins⁴⁸. They are frequently located on transposable elements (Tn4401) located on large plasmid scaffolds⁴⁹. pKpQIL-like plasmids are the most common plasmid backbone on which these enzymes are found^{50,51}. OXA-48 (an extended-spectrum oxacillinase) and NDM (New Delhi Metallo beta-lactamase) enzyme families constitute the other most clinically relevant carbapenemases in KP²⁴.

1.5.3. The outer membrane porins OmpK35 and OmpK36

KP encodes two major outer membrane porins: OmpK35 and OmpK36⁵². They play important physiological roles and facilitate both the influx of small hydrophilic solutes, including nutrients, and the efflux of toxic products across the otherwise impermeable Gram-negative OM⁵³. In keeping with this, deletion of both these porin genes together results in KP attenuation during *in vivo* infection, when administered by intraperitoneal injection in mice⁵⁴. They belong to the family of non-selective OM porins found throughout the Enterobacterales order and substrate selectivity is based on the size of traversing solutes. These features have resulted in OmpK35 and OmpK36 being classified as class I porins, based on multiple sequence alignment and hidden Markov model analysis⁵⁵. Based on early structural work it was calculated that substrates with molecular weights of less than 600 Da could diffuse via OmpK35 and OmpK36 pores across the KP OM⁵⁶. The molecular weight of most β -lactams is less than 550 Da and, as these molecules are hydrophilic in nature, entry into the periplasm can take place via these porin channels. In contrast, other KP OM porins are selective, for example, in the case of LamB, diffusion is restricted to maltodextrins⁵⁷. Here, specificity is conferred by a hydrophobic amino acid tract, at the pore constriction point, with the co-crystal structure of LamB and the maltodextrin, maltotriose, co-localising in this region⁵⁸. A role for porins that transport specific substrates across the OM in antimicrobial resistance is less well understood.

Figure 1.3 Cartoon structures of OmpK35 and OmpK36.



Cartoons of OmpK35 (**A**) and OmpK36 (**B**) reconstructed using atomic coordinates placed in the RCSB Protein Data Bank from data generated by Acosta-Gutiérrez et al⁶¹. The view is extracellular and shows homotrimeric complexes of both proteins. There is significant structural similarity between these porins and other class I porins in the Enterobacterales order. Each monomer provides a single hydrophilic water filled channel through which substrates diffuse into the periplasm from the extracellular space. Substrate selectivity is based on size exclusion in this family, with solutes of <600 Da able to enter via these porins.

Loss or modification of OmpK35 and OmpK36 act to limit carbapenem influx across the outer membrane in CRKP⁵⁹. Whilst the loss of OmpK35 by truncation is common in augmenting the carbapenem resistance in CRKP, OmpK36 expression is rarely lost in CRKP isolates (**Figure 1.2 A&B**). The overall structures of OmpK35 and OmpK36 display similar features to other general porins, such as OmpF and OmpC, in *E. coli*. They are composed of a homotrimeric architecture of 16-stranded β -barrels that traverse the lipophilic OM^{56,60} (**Figure 1.3**). One important structural component in both OmpK35 and OmpK36 is extracellular loop 3 (L3). L3 is not cell surface exposed but folds back into the barrel, forming a constriction zone halfway inside the channel that contributes to the permeability properties, such as size exclusion limit and ion selectivity of the pore⁵⁶. The L3 of OmpK36 contains a conserved central Pro-Glu-Phe-Gly-Gly-Asp (PEFGGD) motif corresponding to the point of maximal constriction in the barrel. Of note, one key difference between the *E. coli* and KP porin homologues is their minimal pore diameter, for example in one study OmpF was found to have a pore minimal radius of 3.1 ± 1.1 Å, whereas OmpK35 was 3.6 ± 1.4 Å. These differences may explain why KP requires porin modification in generating a robust carbapenem resistant phenotype, as the baseline OM diffusion is higher, due to increased diffusion through pores of higher diameter. As these porins play key roles in the physiological transport of nutrients into the cell, any modifications in clinical isolates must balance the diffusion of these substrates with the exclusion of hydrophilic antibiotics.

The precise molecular mechanisms underpinning OmpK36-mediated carbapenem resistance remain poorly understood. To that end, I sought to develop molecular methods for the genetic manipulation of this pressing contemporary pathogen and to build a clinically relevant murine model to evaluate the impact of *ompK36* mutations on *in vivo* fitness. Given this OM porin plays two opposing roles, in facilitating both nutrient uptake in bacterial homeostasis and carbapenem diffusion in antibiotic chemotherapy, it lies at a fascinating intersection in KP adaptation to the modern healthcare environment. I focus this thesis on mutations identified in the high-risk ST258 clonal group as this represents the highest burden of global CRKP disease and the greatest current threat.

Project aims

1. To build, characterise and validate a novel in-house model of severe pneumonia in mice, replicating the mode of KP acquisition observed in Intensive Care patients.
2. To identify and define the precise molecular mechanisms of OmpK36-mediated carbapenem resistance mutations.
3. To use the model generated in (1) and the mutations defined in (2) to establish the fitness cost of OmpK35 and OmpK36 mutations observed in high risk CRKP sequence types.

2. Materials and Methods

2.1. Manuscript screening and data collection

An electronic search was conducted on 15/04/2019 utilising the broad search term string “*Klebsiella* AND pneumoniae AND mouse AND murine AND model”. I restricted the publication period to manuscripts published after (and including) 1990 to facilitate access to primary material. 418 manuscripts underwent primary and secondary screening by myself, Julia Sanchez-Garrido (JSG) and Wen Wen Low (WWL). All screening and data collection was cross-checked for accuracy by independently reassessing each full text by JLCW, JSG and WWL. Any characteristics that were not shown or described in the manuscript were classified as ‘Not stated’. Any measurements described as having been performed, where the data was missing from the publication, were considered as ‘Not stated’ as no conclusions could be extracted from them. If reference was made to a previous manuscript (e.g. inoculation method), I proceeded to access the cited manuscript to enable data collection. If this citation required further references to acquire the information, then data was termed ‘Not stated.’

2.2. Human KP incidence data.

National Health Service (NHS) Hospital Episode Statistics (HES) data is publicly available from NHS Digital and was accessed on 23/03/2021. Hospital episodes recorded under the code B96.1 (*Klebsiella pneumoniae* [*K. pneumoniae*] as the cause of diseases) for the year 2018-2019 were used for the number of reported KP infection episodes. This was normalised to generate an incidence based on publicly available United Kingdom Office for National Statistics population data (available from <https://www.ons.gov.uk/peoplepopulationandcommunity/populationandmigration/populationprojections/datasets/tablea21principalprojectionukpopulationinagegroups>) accessed on 23/03/2021.

2.3. Capsule locus assessment in the EuSCAPE collection

Capsule locus (K-types) data from a structured large European Collection of KP isolates (David et al., 2019²⁴) is available at https://microreact.org/project/EuSCAPE_Kp and was accessed on 23/03/2021.

2.4. Bacterial culture and cloning

All bacterial strains were grown in Luria Broth (10g/L NaCl) supplemented with streptomycin (Sm) 50ug/ml, gentamicin (Gm) 20ug/ml, kanamycin (Kn) 50ug/ml or rifampicin (Rif) 50ug/ml where appropriate. Liquid cultures were grown at 37°C, shaking at 200rpm and stationary phase growth was assumed following overnight growth (i.e. >16 hours). Ertapenem for selection of KPC-2 expressing transconjugants ranged from 0.5mg/L to 2mg/L and was titrated to appropriately to remove non-specific background. LB agar (10g/L NaCl) was supplemented with antibiotics as above and plates incubated overnight at 37°C.

Polymerase chain reaction (PCR) for cloning was carried out using either Q5 High Fidelity 2x polymerase mastermix (NEB, UK) or KOD polymerase Hot Start 2x mastermix (Sigma, UK) following the manufacturer's guidelines. Colony PCR was carried out using Onetaq 2x Quickload mastermix (NEB, UK) following the manufacturer's instructions. PCR product purification, plasmid minipreps and genomic DNA extraction using kits from NEB, following the manufacturers instruction (NEB, UK). All Sanger-sequencing (plasmid or PCR amplicon) was conducted commercially by Eurofins Genomics (Eurofins Genomics, GmBH, Germany).

2.5.1. *Klebsiella pneumoniae* genome editing

KP genome editing was achieved by a scarless (restriction enzyme scar-free), markerless (antibiotic selection marker-free) two-step recombination-based approach, as previously used in *E. coli* and *Citrobacter rodentium*^{62,63}. Recombination was promoted by the expression of lambda *gam*, *exo* and *beta* encoded on pACBSR-Sm⁶⁴, that was introduced into KP_{WT} in previous work. Mutagenesis vectors were constructed in pSEVA612S and conjugated into KP by triparental mating facilitated by *E. coli* pRK2013⁶⁵. 20ul of stationary donor culture, followed by 20ul of stationary *E. coli* pRK2013 culture were allowed to dry on LB plates (without antibiotics) and grown at 37°C for 2 hours. 40ul of KP receiver strain was then overlaid and allowed to dry before a 4 hour incubation at 37°C. Merodiploids (first-step recombinants) were selected on LB plates containing Sm50/Gm20. The second step recombination took place in liquid culture. ISce-I expression, from pACBSR-Sm, was induced by the addition of L-arabinose (0.4% w/v) and after 6 hours of growth at 37°C shaking at 200rpm, the culture was streaked out to single colonies. Colonies were screened by PCR and amplicons were sequenced for confirmation.

2.5.2. Generation of pSEVA612S *glmS* insertion site mutagenesis vectors

Vectors were constructed by Gibson Assembly (NEB, UK) of PCR products. The vector to generate KP PA1-luxCDABE (pSEVA612S-Kp-N2-luxCDABE) was generated in previous work and published in Wong et al. 2019 Nature Communications⁶⁶. This served as a template for the generation of sfGFP and mRFP1 *glmS* insertion vectors. pSEVA612S-Kp-N2-luxCDABE was linearised with primers 1&2 and sfGFP and mRFP1 clones with Bio-fab promoters cloned from pUltra-sfGFP and pUltra-mRFP1⁶⁷ with primers 3&4. luxCDABE was inserted downstream of the Biofab promoter to generate KP Biofab-luxCDABE with the primers (5-8).

2.5.3. Generation of pSEVA612S *ompK35* mutagenesis vectors

Vectors were constructed by Gibson Assembly (NEB, UK) of PCR products. *ompK35* was cloned from the KP_{WT} genome and inserted into linearised pSEVA612S using primers 9-12. The frame shift mutation in *ompK35*_{ST258} was generated by site-directed mutagenesis (SDM) with primers 13&14. SDM PCR products

were recircularised in a one-step kinase, ligase and DpnI reaction (KLD enzyme blend, NEB UK). The *ompK35* deletion vector was generated by inverse PCR using primers 15&16.

2.5.4. Generation of pSEVA612S *ompK36* open reading frame mutagenesis vectors

Vectors were constructed by Gibson Assembly (NEB, UK) of PCR products. *ompK36* from KP_{WT} and KP_{ST258} were cloned into linearised pSEVA612S with primers 17&18. The *ompK36* deletion vector was generated by inverse PCR using primers 19&20. The L3 GD deletion, R127A mutation and LSP deletion were generated by SDM with primers 21-26 using *ompK36*_{ST258} as template. The L3 GD insertion was introduced into *ompK36*_{WT} by SDM with primers 27&28. The *ompK36* 25c>t mutation was a spontaneous PCR error during cloning. The L3 GD insertion was added to this vector by SDM using primers 27 and 28. The 24&25c>t mutation was added to this to the *ompK36*_{WT+GD} vector by SDM using primers 29&30.

2.5.5. Generation of pSEVA612S *ompK36* sfGFP reporter mutagenesis vectors

Vectors were constructed by Gibson Assembly (NEB, UK) of PCR products. The sfGFP reporter was with the *ompK36*_{WT} signal sequence (SS) and *ompK36*_(25c>t) SS were generated with primers 31-34. Codon switches L8 CTG and TTG were created by SDM with primers 35-38.

Generation of *ompK36* 5' untranslated region mutants

Mutants were generated by site-directed mutagenesis as follows: -32(a>t) (P39/P40), -31(a>t) (P39/P41), -30(a>t) (P39/P42), -29(a>t) (P39/P43), -25(a>t) (P44/P45), -23(a>t) (P44/P46), -21(a>t) (P44/P47), -20(a>t) (P44/P48), -18(a>t) (P44/P49), -17(a>t) (P44/P50), -16(a>t) (P44/P51), -14 a>t(a>t) (P44/P52) using leucine 9 TTG sfGFP as the template. The same primer pair (P44/P52) was used to introduce the -14a>t mutation into Leucine 8 CTG-sfGFP, *ompK36*_{WT} and *ompK36*_{WT(25c>t)} vectors.

2.6. Conjugation of pKpQIL into KP strains

pKpQIL-like plasmid was extracted from KP_{ST258} using a bacterial artificial chromosome miniprep kit (Zymo Scientific, CA, USA). 100ng of plasmid was used for electroporation into room temperature competent *E. Coli* DH5α cells prepared according to the protocol published by Tu et al. 2016 Sci Rep⁶⁸. Transformants were selected on LB agar plates supplemented with ampicillin (25ug/ml) and confirmed by colony PCR for KPC-2 (primers 53&54).

pKpQIL-like plasmid was transferred into KP strains by conjugation. Saturated overnight cultures of DH5α pKpQIL were mixed in an 8:1 (v/v) with KP strains containing pACBSR-Sm. The mixture was diluted 1:40 (v/v) in phosphate buffered saline (PBS) and 40ul was incubated on an LB plate at 37°C for 6 hours. The conjugation mixture was that plated on LB Agar plates supplemented with ertapenem (0.5-2mg/L)/Sm 50ug/ml and incubated overnight at 37°C. Transconjugants were confirmed by colony PCR with primers (primers 53&54).

2.7. Outer membrane protein analysis

Isogenic KP strains were grown overnight in either LB or M9 minimal media. Outer membrane proteins were purified according the protocol by Wise et al. 2018 Med Micro ⁶⁹ with no alterations to the published method. 10ug of protein was run by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on a precast 12% TGX gel (Biorad, UK). Gels were stained for protein with Coomassie Brilliant blue R250 (Sigma, UK) according to the manufacturer’s instructions. Gel images were captured on a ChemiDoc XRS+ (Biorad, UK) with appropriate filters.

2.8. Bacterial growth curves and luminescence readings

Saturated overnight LB cultures were diluted 1:1000 into fresh media (either LB or supplemented M9 as described in the figure legends). 200ul aliquots were grown in a 96 well plate, shaking at 200rpm at 37°C. OD_{600nm} measurements were taken every 30 minutes on a FLUOStar Omega (BMG Labtech, UK) plate reader. Luminescence (relative light units, RLU) was obtained for luciferase expressing strains following OD_{600nm} measurements without no emission filter (i.e. open lens).

2.9. Fluorescence assessment on IVIS

Saturated overnight cultures of strains were diluted 1:2000 in PBS. 20ul of suspension was plated onto an agar plate and allowed to dry. Following overnight incubation (16 hours exactly) plates were removed and imaged on an IVIS Spectrum CT imaging platform (1 second exposure, Ex465nm/Em520nm). Fluorescence over the area of the spot was quantified using Living Image Software v5.4.3 (Perkin Elmer).

2.10.1. Carbapenem MICs by broth dilution

The MIC of strains was assessed by broth microdilution in biological triplicate according to the ISO 20776-1:2019 standard and the median value taken as the final MIC. In Chapter 4 this was conducted at Public Health England (PHE, Colindale, UK) and in Chapter 5 in the laboratory of Gian Maria Rossolini (University of Florence, Italy).

2.10.2. Carbapenem MICs by agar dilution

Plates for agar dilution MIC determination were made in accordance to CLSI guidance⁷⁰ using Mueller-Hinton Agar (Merck, UK) supplemented with meropenem, ertapenem or imipenem. Saturated overnight cultures of test isolates were diluted in 0.8% saline and 20ul (10⁴-10⁵ CFU) plated and incubated for 18 hours at 37°C.

2.11.1. Animal work

8-10 week old, female, 18-20g BALB/c mice were purchased from Charles River, UK. All animal work took place in the institution animal facility (Association for Assessment and Accreditation of Laboratory Animal

Care accredited) under the auspices of the Animals (Scientific Procedures) Act 1986 (PP7392693). Work was approved locally by the institutional ethics committee.

2.11.2. Housing and Randomisation

Upon arrival animals were independently randomized into cages of 5 animals and housed for an acclimatisation period of 1 week. Mice received food and water *ad libitum* and were housed in a 12hour/12hour light dark cycle. Identification of animals within groups was achieved by ear notching without anaesthesia at least 24 hours in advance of procedures.

2.11.3. Intratracheal administration of inoculum and infection

Anaesthesia was induced by intraperitoneal injection (BD Microlance 27G 13mm needle) of 80mg/kg ketamine and 0.8mg/kg medetomidine. Pre-intubation body temperature was maintained by contact with a heat mat (Harvard Apparatus, U.K) and eye lubricant (2.0mg/g carbomer, Alcon UK) was applied.

Intubation was achieved as previously described⁶⁶by placement of a 21G catheter (21G IV peripheral catheter (Insyte) BD Medical) using a fiberoptic intubation set (Kent Scientific) following the enclosed instructions.

Inoculum (dose defined in graphs) was prepared by dilution of saturated overnight cultures into phosphate buffered saline (PBS) (total inoculum volume 50ul) and following inspiration it was dispersed with 2 x 200ul air flushes. An aliquot of inoculum was enumerated for each administration and all inoculums were ±10% of the stated figure.

Mice recovered at 32°C (Warm Air System, Safety Cabinet Version red, VetTech, UK) until spontaneous movement and received 0.8mg/kg subcutaneous (BD Microlance 27G 13mm needle) atipamezole to reverse the α-agonist into the neck scruff.

At the experimental end-point animals were anesthetized with ketamine 100mg/kg and 1mg/kg medetomidine by intraperitoneal injection (BD Microlance 27G 13mm needles). Following the induction of anaesthesia, blood was collected by a transdiaphragmatic inferior approach cardiac puncture (BD Microlance 25G 25mm needles) and animals were then humanely killed, under anaesthesia, by cervical dislocation.

An aliquot of blood (20ul) was taken and diluted into 180ul of hypotonic lysis solution (1mM EDTA in water) for enumeration of bacterial counts. The rest was placed into a microtainer (BD Microtainer for serum collection, BD Medical) and allowed to clot for 1 hour. This was then spun according to the manufacturer’s guidance and aliquoted into cryobanking tubes (Greiner Bio-One) and frozen at -80°C until analysis.

Lungs were dissected out, weighed and then homogenised in gentlemacs C-tubes (Miltenyi Biotec) containing 3 ml complete RPMI media (RPMI supplemented with 10% heat inactivated FBS, 10 mM

HEPES and 1mM Sodium Pyruvate) using a gentleMac Octo dissociator (Miltenyi Biotec) using the program m_lung_1 twice.

Bacteria were enumerated from the lung homogenate and blood by dilution in PBS and plating onto solidified LB agar containing 50ug/ml rifampicin (Merck, UK). In competition assays plates were UV transilluminated to determine colony fluorescence.

2.11.4. In vivo imaging

Mice were anaesthetised, intubated and administered inoculum and 2D or 3D bioluminescent imaging took place on an IVIS SpectrumCT live imaging platform (Caliper Life Sciences, MA, USA). Animals underwent a MicroCT scan and acquisition of bioluminescence data at three emission wavelengths (500nm/520nm/540nm). Images were reconstructed in Living Image 4.5.5. with the software's preset values (Caliper Life Sciences, MA, USA).

2.11.5. Antibiotic delivery

We combined the meropenem (100mg/kg) with cilastatin (100mg/kg) (Merck, UK), a renal dihydropeptidase inhibitor, to reduce the *in vivo* metabolism of meropenem as the murine enzyme isoform has increased activity against this carbapenem compared to human enzyme. Drugs were diluted in dH₂O. dH₂O alone was administered to vehicle control animals. Doses were delivered at 6 hourly intervals as previously employed in a murine carbapenemase producing KP model⁷¹. The drug mixture was delivered by intraperitoneal infection (BD Microlance 27G 13mm needles) to animals at 6 hourly intervals and the iliac fossa used was alternated between doses.

2.11.6. Cytokine bead assay

Serum IL-6, CXCL-1 and IFN- γ were assessed using a custom-made mouse panel 13-plex kit (LEGENDplex, BioLegend) following the manufacturer's instructions. Cytokine levels were acquired using a FACSCalibur flow cytometer (BD Biosciences), and analyses were performed using LEGENDplex data analysis software (BioLegend). A biological repeat was considered valid only if at least 80% of all the samples run were above the limit of detection; any values below the detection limit within a repeat such as this were assumed to be the lowest value detectable by the assay for statistical analysis. Values below the detection limit in graphs were displayed as 1 for visualisation.

2.11.7. Neutrophil quantification in lung homogenate by flow cytometry

1 ml of complete RPMI supplemented with penicillin and streptomycin solution (final concentration of 100 U and 100 μ g/ml respectively), Liberase TM (0.13 mg/ml final, Roche) and DNaseI (10 μ g/mL; Sigma-Aldrich) was added to the lung homogenate after removal of 30 μ l for lung CFU calculations. The homogenate was then incubated for 40 mins on a shaker at 37 °C to allow for enzymatic tissue disruption and cell dissociation. After this incubation, the samples were placed on ice to interrupt the enzymatic

digestion and the enzymes were further inhibited by adding EDTA (final concentration 10 mM; Gibco). The samples were run through a 70 μ m cell strainer to obtain a uniform single-cell suspension, spun down and resuspended in 600 μ l of complete RPMI. From here onwards, steps were carried out on ice to preserve cell viability.

For staining, $\sim 5 \times 10^6$ cells (around 120 μ l) from each sample were added per well in a 96-well V-bottom plate. Dead cells were routinely excluded with Zombie Aqua Fixable Dead Cell Stain (ThermoFisher Scientific). Single cell suspensions were incubated with Fc block (Miltenyi Biotec) in FACS buffer (1% BSA, 2mM EDTA in DPBS, Sigma), followed by staining with anti-CD11b-PerCP-Cy5.5 (#45-0112-82, ThermoFisher Scientific) and anti-Ly-6G-FITC (#551460, BD Pharmingen) in FACS buffer for 30 min, all at 4°C in the dark. Fluorescence minus one (FMO) controls and a fully unstained sample were always included as controls. Stained cells were washed in FACS buffer and fixed for 20 mins at room temperature with 1% paraformaldehyde in PBS; fixed cells were kept in the dark at 4°C until analysis, usually the following day. On the day of the analysis, single stain controls for compensation were prepared using VersaComp beads (Beckman Coulter) and ArC Amine Reactive Compensation beads (ThermoFisher Scientific) and a known volume of CountBright absolute beads (ThermoFisher Scientific) was added to each sample before running the samples. Flow cytometry analysis on 50000 live cells was performed on a BD LSRFortessa cell analyzer (BD Biosciences). Data were analysed using FlowJo software (Tree Star). Neutrophils were defined as CD11b+ Ly6G+ live cells and absolute numbers of cells in the sample were calculated using the numbers of CountBright absolute beads counted following the manufacturer's instructions.

2.12. Software and statistics

Graphs and statistical analysis was carried out in GraphPad Prism v9.0.0 for Mac (GraphPad Software, San Diego, California USA, www.graphpad.com). Data were analysed for normal distribution (based on D'Agostino-Pearson or Shapiro-Wilk normality tests) and if not normally distributed a base 10 logarithmic transformation was applied before statistical analysis. When normality was not achieved by transformation, a non-parametric test was applied. Statistical tests applied for each analysis are described in associated figure legends.

Custom images of mice were made with Biorender (created with BioRender.com). Images were edited and compiled into figures in Affinity Designer v1.8.4. for MacOS (Serif Europe Ltd). The thesis was compiled in Affinity Publisher v1.10.5 for MacOS (Serif Europe Ltd).

2.13. Primers used in this study

All primers were synthesized by Sigma, UK.

Primer	Sequence
1	GCTGCCGACGGCGGCCCT
2	CCTTTGCAAGATAGGATGCTTTAC
3	ATCCTATCTTGCAAAGGCGATAGAGTATTGACTTCG
4	AGGGCCGCCGTCGGCAGCCGCGAAGTAATCTTTTCG
5	GGAGTTTAAGGAGGTAAAAA
6	TAAGGATCCAACAGGGTTC
7	TTTAAGGAGGTAAAAAATGACTAAAAAATTCATTCAATTAAAC
8	GTGATGCTTTTCGAATTTATTTAATAAGGATCCAACAGGGTTC
9	ATTACCCTGTTATCCCTATAC
10	TAGGGATAACAGGGTAATC
11	GGATTACCCTGTTATCCCTAATGAAGCGCAATATTCTGGC
12	TATAGGGATAACAGGGTAATTTAGAACTGGTAAACGATACCC
13	AGCACGTCTGGACCACCAATGGC
14	CGCCCGACCATTTTTCCATAGAAGTCCAG
15	TCTGCAGTACACCCCTTC
16	CATTATTTATTACCCTC
17	GGATTACCCTGTTATCCCTAGGCCTAATTGATTGATTAATAGTCG
18	TATAGGGATAACAGGGTAATAGCCCCACAGGTTGACCAGC
19	GTTATTAACCCTCTGTTTG
20	GTTGCAAGCTGCATAAC
21	GGCGACACCTACGGTTCT
22	GCCGAATTCCGGCAGAAC
23	GTCAGCGGCGAAGGCGCT
24	GTCAGCGGCGAAGGCGCT
25	CCTGCAGTCCGCTGCTAACGGCG
26	AAGTTGTCAGAACCGTAG
27	CGGCGACGGCGACACCTACGGTTC
28	CCGAATTCCGGCAGAACG
29	TACTGTCCCTTTTGGTACCGG
30	GAGGGTTAATAACATGAAAGTTAAAG
31	GTTGCAAGCTGCATAAC
32	CGCATTTGCTGCGCCTGCTAC
33	GTAGCAGGCGCAGCAAATGCGCGTAAAGGCGAAGAGCTG
34	GTTATGCAGCTTGCAACCGCGAAGTAATCTTTTCG
35	TTAACTTTTCATGTTATTAACCCTCTGTTTGTTATG
36	AGTACTGTCCCTGCTGGTACCGG
37	AGTACTGTCCCTGCTGGTACCGG
38	TTAACTTTTCATGTTATTAACCCTCTG
39	GCATATAACAAACAGAGGGTTAATAAC
40	CTTTAATTATGCCACTGC
41	CTTATATTATGCCACTGC
42	CTATTATTATGCCACTGC
43	CATTTATTATGCCACTGC
44	CTTTTATTATGCCACTGC
45	GCTTATAACAAACAGAGGGTTAATAAC
46	GCATTTAACAAACAGAGGGTTAATAAC
47	GCATATTACAAACAGAGGGTTAATAAC
48	GCATATATCAAACAGAGGGTTAATAAC
49	GCATATAACTAACAGAGGGTTAATAAC
50	GCATATAACATACAGAGGGTTAATAAC
51	GCATATAACAATCAGAGGGTTAATAAC
52	GCATATAACAACTGAGGGTTAATAAC
53	TTCATGCGCTCTATCGGCGATA
54	GCATAGTCATTTGCCGTGCCAT

Chapter 3. *In vivo Klebsiella pneumoniae* models.

3.1. Introduction

Animal models have been widely employed to study infectious diseases from the nematode *Caenorhabditis elegans*⁷² to non-human primates⁷³. Whilst some animals are particularly suited to modelling certain human diseases, for example ferrets and influenza A infection⁷⁴, the laboratory mouse, *Mus musculus*, is the most commonly utilised species across the field. Their dominance, as a primary experimental tool, is in part driven by their low cost, fast reproductive time with large litters, straightforward husbandry and ease of housing⁷⁵. Additionally, whilst clear genetic differences are present, the laboratory mouse shares 90% of the genes with humans and therefore, the function of genes discovered in mice can be often translated into humans and vice versa⁷⁶. Clarence Cook Little generated the first inbred mouse line (DBA mice) and such lines provide the benefit of an identical genetic background⁷⁷. This has had enormous benefits in mouse-based work, as heterogeneity in host genetics is abolished, reducing experimental variability and allowing for smaller numbers of animals to be used in statistically testing a hypothesis. As our understanding of genetics has proceeded and both genetic sequencing and analytical tools have become increasingly available, an opposing trend is now being exploited. In mice, this has taken the form of the Cross Collaborative (CC) collection⁷⁸. The CC collection is a multi-parental recombinant mouse strain collection, derived from eight laboratory inbred strains, and differences in host responses, to the same infection, can be used to identify susceptibility or resistance loci⁷⁹. Despite the potential shortcomings of murine models and physiological differences⁸⁰, they have proven invaluable in studying infectious disease and unravelling molecular bacterial pathogenesis.

The lower respiratory tract represents the site with the highest burden of bacterial infectious diseases globally⁸¹. It is also the leading primary site of infection resulting in severe illness necessitating intensive care treatment^{12,82}. Of particular concern are hospital-acquired lung infections, including hospital-acquired pneumonia (HAP) and ventilator-acquired pneumonia (VAP) as infection is frequently complicated by pathogen-associated antimicrobial resistance and high mortality⁸¹. One key contemporary pathogen associated with both these nosocomial infections is *Klebsiella pneumoniae* (KP). Whilst intra-alveolar damage results in impaired pulmonary function, sepsis and septic shock frequently complicate infection at this primary site and bacteraemia occurs following dissemination into the blood⁸³. The ongoing COVID-19 pandemic has demonstrated the damaging nature of an excessive and injurious host response with persistent life-threatening lung injury (acute respiratory distress syndrome, ARDS), despite pathogen clearance by the immune system⁸⁴. In advance of the global coronavirus pandemic, the leading cause of

ARDS was bacterial pneumonia and, whilst effective vaccines may prevent this pathology in the case of SARS-CoV2, no prophylactic therapies exist for KP. In fact, the only licensed vaccine for a bacterial lung pathogen is the pneumococcal conjugate vaccine conferring protection to *Streptococcus pneumoniae*⁸⁵. Whilst this is a common community-acquired pneumonia⁸⁶ there is a notable lack of vaccines for any of the WHO Critical Priority organisms, which includes carbapenem-resistant *Pseudomonas aeruginosa*, carbapenem-resistant *Acinetobacter baumannii* as well as third generation cephalosporin and carbapenem-resistant KP. These organisms form the burden of hospital-acquired infections which result in high morbidity, mortality and economic burden^{43,81}. Instead, the cornerstone of treatment is antibiotic chemotherapy, however, the effectiveness of key antibiotic classes, such as carbapenems, is threatened by rapidly mounting levels of resistance in clinical isolates⁸¹.

Genetic engineering in both mice and bacteria is now routine and, together with straightforward gene deletions, more complex genetic alterations such as gene substitutions or the introduction of point mutations can now be reliably achieved in both mammalian and bacterial genomes. The overarching aim of this thesis is to investigate chromosomally-encoded carbapenem resistance mutations in KP and to contextualise them in terms of their molecular mechanism as well as their impact on virulence *in vivo*. In order to assess the latter, I started this project by generating an in-house KP mouse infection model in which I aimed to recapitulate as many elements of HAP and VAP as possible. To that end, in this results chapter I complete an extensive critical review of the published literature, to provide a broad high-level overview of the methodologies and reagents that have been used to study experimental KP infection in the lungs of mice. This includes a detailed analysis of the mouse host, the KP strains used for infections together with the methods used to induce infection and the variables assessed as outcomes. I then use this synthesis of knowledge to build a new KP model which I validate with techniques including *in vivo* imaging. Alongside this validation, I demonstrate the ability to genetically engineer KP and generate fluorescently tagged strains, by inserting genes encoding fluorescent proteins, at a silent genomic insertion site, as tools for future experimental work in this thesis.

3.1.1. Specific chapter aims:

1. To utilise a structured literature review to illustrate the current practice in modelling KP pulmonary infection in preclinical murine models.
2. To develop and validate a new severe KP lung infection model in mice.

3.2. Results

3.2.1. Manuscript curation and data collection using a PICO framework

In order to identify *in vivo* models that had been employed to investigate KP pulmonary infection in mice, I conducted a Pubmed/MEDLINE search on 15/04/2019. This identified 418 publications. The manuscript selection was further refined by applying two rounds of screening to exclude manuscripts not meeting our desired criteria (**Figure 3.1A**). In the primary screen, *in vivo* models in which KP was inoculated at an extrapulmonary primary site were excluded. If this information was not available in the manuscript's abstract, I proceeded to seek this information in the main body of the work. All publications also had to comprise primary research and this initial screen excluded 260 manuscripts. I noted that exclusions largely occurred as KP infection was initiated at a site outside the lungs. Here, the majority of work consisted of KP murine thigh infections that are widely employed as pharmacokinetic models to assess the efficacy of antimicrobial agents. In the secondary screen I excluded 6 further manuscripts. Two manuscripts were published in a language other than English (both Chinese) and four did not have accessible full text copies. In the case of the latter, I attempted to gain access to the works by contacting both the corresponding author and publisher and in all four cases I was unable to obtain the material. Following these exclusions, 152 manuscripts were identified for analysis. I acknowledge that I was aware of publications that would have met the inclusion criteria but were not identified using this systematic search strategy. However, to avoid biasing the content of this analysis towards my areas of focus in KP pathogenesis, I did not retrospectively add manuscripts into the collection.

I systematically collected data from all 152 manuscripts by adapting a research framework, termed PICO (Population, Intervention, Comparison, Outcomes), which is usually employed to answer a clinical or healthcare related query in evidence-based medicine⁸⁷. Data were collected from each manuscript and recorded into a database. This was then independently cross-checked to ensure accuracy by two further researchers and where discrepancies occurred a consensus was reached. Where no consensus could be reached, data was recorded as 'Not stated' or 'Not available'. To that end, I collected data under four domains that were designed to collect pertinent information regarding the host (mice), infecting organism (KP), experimental infection methods and outcome measures of infection (**Table 3.1**). In the first domain I assessed the heterogeneity of study populations including data on mouse sex, age and breed, as well as genetic alterations and conditioning prior to infection. In work where genetically altered (GA) mice were used to evaluate the function of a host (mouse) gene I collected and summarised infection outcome data. KP strains were assessed in the Intervention and Comparisons domain including information regarding strain identity (name), capsule (K) type and inoculation method. KP genetic mutants were assessed in

Figure 3.1 Manuscript selection criteria and features of mice used for experimental infection.

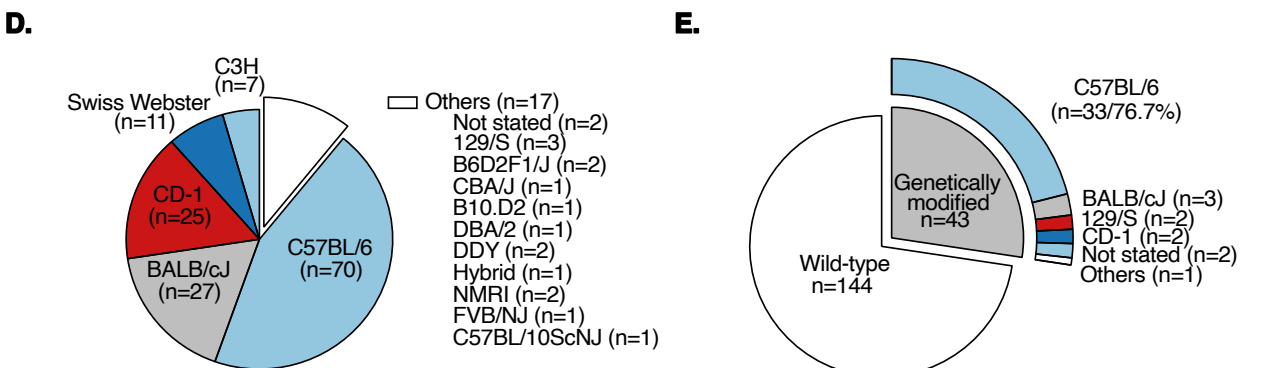
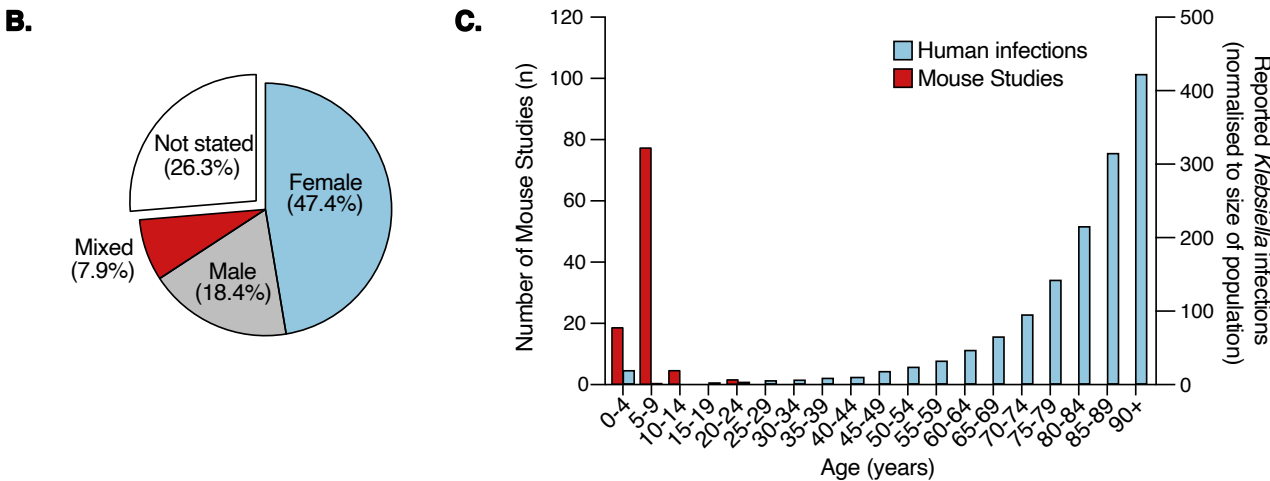
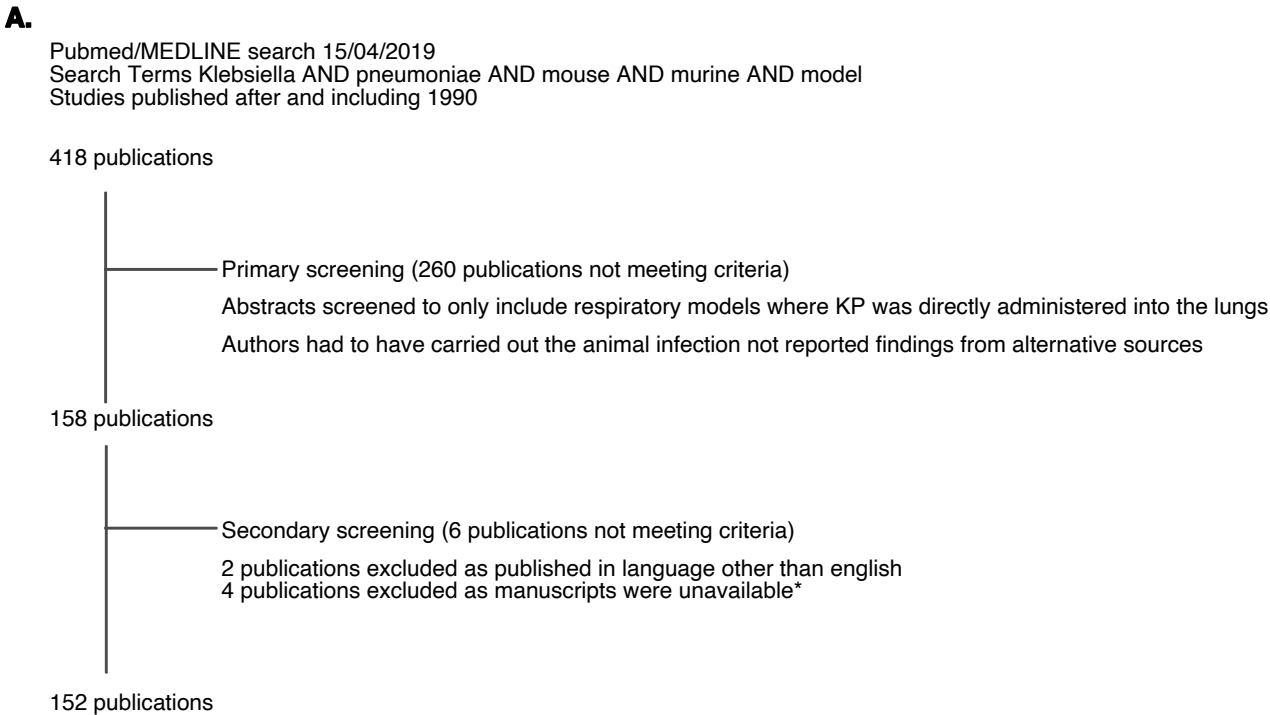


Figure 3.1 Legend

A. An unbiased search strategy with broad search terms was employed to identify 418 peer-reviewed manuscripts. This underwent primary and secondary screening resulting in 152 manuscripts which were included in the final dataset; the majority of manuscripts were excluded because KP was administered to extrapulmonary primary sites. *Manuscripts were considered 'unavailable' if we were unable to access the full text after contacting both the corresponding author and the original publisher.

B. The distribution of mouse sex used in experimental KP infections. Sex description was not stated in 26.3% (40/152) of studies; where sex was stated, females were most commonly used.

C. A comparison of mouse age at infection with the incidence of KP infections in the human population (2018-2019 United Kingdom data). Mouse ages underwent a linear conversion into human age (9 mouse days equating to 1 human year) and the number of studies were presented as bar plots (red bars, left axis indicating the number of studies). The number of reported human KP infections (normalised to the size of the population) are presented in blue bars (right axis indicating the incidence of reported KP infections). The age range at which mice were infected was described in 69.7% (106/152) of studies. Most mice were infected at a mean age of 7 weeks, with 82.0% (87/106) of studies using mice at a mean age between 6-10 weeks.

D. 15 different mouse breeds were identified in the 152 manuscripts, with C57BL/6 mice being the most frequently used breed.

E. Wild-type mice were most frequently used and when genetically altered (GA) mice were infected, C57BL/6 mice were the most common parental breed. The outer ring indicates the proportion of parental mouse breed used for genetic modification.

further detail and outcome measures of infection were compared when infection with isogenic KP mutants were used to evaluate the impact of disruption of bacterial genes. Finally, I focused on the experimental outcomes that were measured as variables to assess and quantify infection. This included an in-depth analysis of the KP bacterial burdens and host immune responses (cytokines and chemokines). I assessed both the site (biological material) and the time at which samples were collected post KP inoculation. The measurement of physiological assessments of infection (e.g. heart rate) are not well defined in mice, and I explored their use in this collection of manuscripts.

3.2.2. Population domain I: mouse sex, age, weight and breed.

Mouse sex was reported in most studies but was not available in 26.3% (40/152) of manuscripts (**Figure 3.1B**). Female mice were used in 47.4% (72/152) of studies, males in 18.4% (28/152) and a mixture of sexes in only 7.9% (12/152) of the manuscripts. The bias towards experimental KP infection in female mice may represent the practicalities of animal housing, as male co-housing often results in aggression with in-cage injury from fighting⁸⁸. When both sexes were included, this was usually in the case of GA mice. Animals used in these studies tended to be derived from locally housed breeding colonies, instead of licensed commercial breeders of mice for use in research, which may have limited the availability of single sexes for experiments.

Mouse age, without accompanying weight data, was provided in 49.3% (75/152) of studies. Mouse weight, independent of mouse age, was reported in 13.2% (20/152) and the combination of age and weight was provided in 20.4% (31/152) of studies. In 17.1% (26/152) of cases, I was unable to locate data pertaining to either metric. In total, 106 studies reported the age of experimental animals and infections were carried

Table 3.1 An adapted PICO approach for the systematic collection of data from manuscripts included in this study.

PICO approach to investigate experimental <i>K. pneumoniae</i> lung infections in mice	
POPULATION (covers mouse population used for infections)	
Subdomain	Additional Information
Mouse sex	Female/Male/Mixed or Not stated
Mouse age	Age range in weeks at infection
Mouse breed	Mouse breed used for infections
Mouse genetics	Mouse genetic modification including host and pathogen outcomes of infection.
Mouse induced co-morbidity	Co-morbidity induced in mice prior to experimental infection, method of induction, purpose of induction including host and pathogen outcomes of infection.
INTERVENTION (covers <i>K. pneumoniae</i> strains and inoculation method)	
Subdomain	Additional Information
<i>K. pneumoniae</i> strain	<i>K. pneumoniae</i> strain name if stated
<i>K. pneumoniae</i> capsule type	<i>K. pneumoniae</i> capsule (K) type if stated
Inoculation methodology	Method utilised to introduce bacteria into the lungs of mice
Inoculation dose	Dose of <i>K. pneumoniae</i> used for experimental infection
Anaesthesia	Anaesthetics used to facilitate animal procedures including drug combinations.
COMPARISON (covers <i>K. pneumoniae</i> mutants)	
Subdomain	Additional Information
<i>K. pneumoniae</i> genetic mutants	Genetic mutations induced in <i>K. pneumoniae</i> including origin of mutations (spontaneous/genetically engineered), strain background for mutations and outcomes of infection.
OUTCOMES (covers outcome variables assessed in infections)	
Subdomain	Additional Information
<i>K. pneumoniae</i> bacterial burdens	Bacterial burden assessment including time post infection, site of investigation (lungs/blood/spleen/liver/bronchoalveolar fluid or other site) stratified by strain used for infection.
Survival endpoints	Experimental endpoints for mice including mortality assessment.
Host immune assessment (cytokines/chemokines)	Cytokine/chemokines/inflammatory mediator assessment including time post infection, site of investigation (lung homogenate/bronchoalveolar fluid/blood/serum) stratified by measured variable.
Physiological endpoints	Experimental endpoints for mice focusing on perturbation in physiology following infection.

out in 6-10-week old mice in 82.0% (87/106) of the studies. Mice were consistently infected at a young age and the peak age at which KP infection took place was 7-weeks-old (**Figure 3.1C**).

I wanted to draw comparisons between the age of experimental animals and the age of the human population infected with KP. When mouse age data was not supplied, but weight data was available, I inferred the age of mice by using breed-specific body weight chart/age nomograms (<https://www.jax.org/search?q=body%20weight>, or growth charts in <https://www.criver.com/products-services/find-model>). This approach allowed me to analyse the data from 126 individual studies. I started by converting mouse age

to human age by employing a simplified (linear) formula that assumes that 9 mouse days is equivalent to approximately 1 human year⁸⁹. As an example, applying this formula to 6–10-week-old mice results in a human age equivalence of 4.7-7.8 years. I then used the United Kingdom National Health Service (NHS) Hospital Episode Statistics (HES) data available from NHS Digital to determine the number of hospital episodes that were recorded under the code B96.1 (*Klebsiella pneumoniae* [*K. pneumoniae*] as the cause of diseases) by age group reported in 2018-2019. As these data represent the raw number of reported KP episodes in each age group and do not take into account the size of the population within each age bracket, I normalised these counts to the estimated population size in each age range. The population and age distribution data were accessed from the UK Office for National Statistics population report (2018). I then plotted the mouse age (converted into human years) and human incidence data side-by-side (**Figure 3.1C**). This demonstrated that whilst the peak of infections in humans occurs at >90 years old, experimental mice are infected at the opposite side of the age spectrum. Of note, most experimental studies infected mice in human-adjusted ages with the lowest observed incidence of human disease. When older mice were used for infections, this was usually as the area of study was the impact of obesity on KP infection and target weight required an older age to establish the phenotype⁹⁰. Nevertheless, the oldest mice used in experiments were 7.5 months old (30 weeks), equivalent to only 23.3 years of age in a human (**Figure 3.1C**).

15 different mouse breeds were used in experimental KP infections and in two manuscripts the breed was not stated. Inbred mouse breeds made up the majority of animals used in KP animal studies with C57BL/6 and BALB/c mice used in 46.0% (70/152) and 17.8% (27/152) of studies, respectively (**Figure 3.1D**). Two outbred mouse strains were also well represented with 16.4% (25/152) of studies using CD-1 mice and 7.2% (11/152) using Swiss Webster mice. Three further studies employed NMRI mice, which represent another outbred mouse line. Typically, individual publications used a single mouse breed although in seven cases more than one breed was used in a single manuscript. All seven of these manuscripts used GA animals to study the role of a host factor during KP infection and its impact on infection outcomes.

I identified four occurrences in which KP infection took place in C3H/HeJ mice, a sub-strain of C3H mice carrying a natural loss-of-function Toll-like Receptor 4 (TLR4) mutation. Whilst this is a spontaneous mutation and not genetically engineered, it is known to render C3H/HeJ mice more susceptible to Gram-negative infections^{91,92}. In addition, DBA/2 and B10.D2 mouse strains, which both carry an identical loss-of-function mutation in complement factor 5 (C5) were each used on a single occasion. Whilst loss-of-function mutations in C5 are rare in humans they are well described and lead to an increased susceptibility to *Neisseria meningitidis* meningitis⁹³.

3.2.3. Population domain II: Genetic alterations and experimental pre-conditioning.

A large proportion of manuscripts used KP as a model Gram-negative organism to investigate the role of immune components in host defence. I identified 43 manuscripts in which GA mice were used to investigate a gene’s function in the host’s response to KP infection (**Figure 3.1E**). 76.7% (33/43) of GA mice were constructed on a C57BL/6 background, which underlies our overall reliance on this breed in KP infection models (**Figure 3.1E**). Genetic alterations were made in other breeds on rare occasions including in BALB/c (n=3), 129/S (n=2), and CD-1 (n=2) mice.

I looked at all the manuscripts (43) that utilised GA mice and constructed a summary table, which includes the genetic mutations, the mouse breed and infecting KP strain and the impact of the mutation on the infection outcomes. I was able to subdivide this list into mutations that were disadvantageous (33 occurrences, **Table 3.2**), neutral (4 occurrences, **Table 3.3**) or advantageous (9 occurrences, **Table 3.3**) to the host following KP infection. In two cases (*Nlrc4*^{-/-} and *Ptx3* overexpression) the effect of these genetic alterations was variable with groups reporting different or inconclusive findings^{94–96}. Notably, the importance of IL-17 signalling in the protective immune response against KP was strengthened by the use of two different genetic targets (deletion of the IL-17A cytokine or its receptor⁹⁷) by three different research groups^{95,97–99}. IL-17 responses are key in mucosal immunity, but can also result in excessive inflammation making them a target for immune modulation^{100,101}; this further highlights the robustness of the results in these KP mouse models, where impairment of IL-17 signalling leads to increased bacterial burdens and host mortality (**Table 3.2**). Most studies involving GA animals were performed in C57BL/6 mice (**Figure 3.1D**) and on only one occasion, in the case of TLR4, were two different mouse breeds (used by different groups) used to investigate the role of the same host gene on KP infection outcomes^{97,102}. Both groups demonstrated impaired lung clearance and lower lung bacterial burdens when this gene was disrupted, correlating with what has been observed in Gram-negative infections in humans^{103,104}.

KP infection is associated with co-morbidity in humans with diabetes, excess alcohol consumption and immunodeficiency reported as underlying conditions predisposing to infection¹⁰⁵. I screened the 152 manuscripts and identified 12 experimental conditioning regimes utilised to induce co-morbidities that were introduced into the murine infection models. I summarise this in **Table 3.4** and subdivide these into two major groups; co-morbidities induced to investigate the role of a host co-morbidity on KP infection outcomes and co-morbidity induced to permit infection or mimic human physiology. In the former, alcohol consumption was used in five different manuscripts and universally worsened the outcomes of infection (**Table 3.4**). The use of different KP strains, mouse breeds and alcohol administration routes and regimens

Table 3.2 Disadvantageous mouse genetic alterations, including mouse and KP strain with outcomes of infection.

Host Genetic Mutation	KP/mouse strain	Effect on <i>Klebsiella pneumoniae</i> infection	Reference(s)
MUTATIONS DISADVANTAGEOUS TO HOST FOLLOWING INFECTION			
5-Lox^{-/-} (5-lipoxygenase) Also known as: Arachidonate 5-lipoxygenase, ALOX5 and 5-LOX. Notes: Non-conditional knock-out	ATCC43816 129/SvEv	Increased bacterial burdens in lungs and blood. Increased host mortality.	Baillie et al., 1996
Asc^{-/-} (Apoptosis-associated speck-like protein containing A CARD) Also known as: Pycard Notes: Non-conditional knock-out	ATCC43816 Not stated	No difference in bacterial burdens. Reduced inflammatory response and increased host mortality.	Willingham et al., 2009
C3^{-/-} (Complement component 3) Notes: Non-conditional knock-out	TOP52, ATCC43816 C57BL/6	Increased bacterial burden (Not required for suppression of growth of second <i>K. pneumoniae</i> inoculum in a two-hit model of recurrent bacterial aspiration).	Ben-David et al., 2005 Rosen et al., 2016
CCR2^{-/-} (C-C chemokine receptor type 2) Also known as: CD192 Notes: Non-conditional knock-out	' <i>K. pneumoniae</i> serotype 2' C57BL/6	Impaired bacterial clearance. Increased host mortality.	Herold et al., 2011
Cnlp^{-/-} (Cathelin-like protein) Also known as: Camp, Cramp Notes: Non-conditional knock-out	ATCC43816 C57BL/6	Impaired lung bacterial clearance and increased bacterial dissemination. Increased host mortality.	Kovack et al., 2012
db/db (Leptin receptor db mutation) Notes: Homozygous mice that suffer from severe obesity and are used as a model for type 2 diabetes.	ATCC43816 C57BL/6 (C57BLKS/J)	Increased bacterial burdens in lungs, increased spleen bacterial counts at 48hpi. Impaired immune response.	Ubags et al., 2014 Ubags et al., 2016
Kitt^{wt/wt} (KIT tyrosine protein kinase) Also known as: CD117 or Scfr Notes: Non-conditional knock-out in the protooncogene KIT that causes lack of mast cells and anaemia.	KP1415 Not specified	Increased bacterial burdens. Decreased neutrophils.	Malaviya et al., 1996
Kitt^{wt/sh}/Kitt^{wt-sh} (KIT tyrosine protein kinase) Also known as: CD117 or Scfr Notes: Non-conditional knock-out in the protooncogene KIT that causes lack of mast cells.	ATCC43816 C57BL/6	Increased host mortality.	Sutherland et al., 2008
LBP^{-/-} (Lipopolysaccharide Binding Protein) Notes: Non-conditional knock-out.	ATCC43816 C57BL/6	Increased bacterial burdens. Impaired immune response and increased host mortality.	Hemmila et al., 2005 Taddonio et al., 2015
limg^{-/-} (Interferon gamma) Notes: Non-conditional knock-out	ATCC43816, <i>K. pneumoniae</i> -396 (serotype K2) C57BL/6	Impaired bacterial clearance. Intranasal immunisation using heat-killed bacteria unaffected. Decreased protective effect of CpG oligodeoxynucleotide in <i>K. pneumoniae</i> infection. Increased host mortality.	Moore et al., 2002 Chen et al., 2011 Deng et al., 2014
Ighm^{-/-} (Immunoglobulin mu chain C region) Notes: Non-conditional knock-out	ATCC43816, <i>K. pneumoniae</i> -396 (serotype K2) C57BL/6	Increased lung and spleen bacterial burdens in non-immunised mice (not directly compared). Intranasal immunisation with heat-killed bacteria still protective against autologous challenge (same serotype), but less effective than in wild-type mice, and heterologous challenge.	Chen et al., 2011
Lcn2^{-/-} (Lipocalin-2) Also known as: NGAL, Scn, and 24p3 Notes: Lipocalin-2 is able to bind the siderophore enterobactin, preventing its uptake and use by bacteria.	ATCC43816 C57BL/6	Increased bacterial dissemination and lung and spleen bacterial burdens. No effect if <i>K. pneumoniae</i> cannot utilise siderophores (Δ tonB). Increased host mortality.	Chan et al., 2009 Bachman et al., 2011 Holden et al., 2016

Table 3.2 Disadvantageous mouse genetic alterations, including mouse and KP strain with outcomes of infection (continued).

Host Genetic Mutation	KP/mouse strain	Effect on <i>Klebsiella pneumoniae</i> infection	Reference(s)
MUTATIONS DISADVANTAGEOUS TO HOST FOLLOWING INFECTION			
Ly49q^{-/-} (Ly49 C-type lectin-like receptor) Notes: Non-conditional knock-out in an inhibitory NK receptor	Strain not specified C57BL/6	Increased bacterial burdens. Increased immune cell infiltration.	Margraf et al., 2018
Il-6^{-/-} (Interleukin-6) Notes: Non-conditional knock-out	ATCC43816 C57BL/6	Increased bacterial burdens. Increased host mortality.	Sutherland et al., 2008
Il-10 overexpression (Interleukin-10) Notes: A bi-transgenic mouse with allows doxycycline-regulated and lung specific overexpression of human IL-10 (hIL-10)	ATCC43816 FVB/n	Increased bacterial burdens in lungs and blood Impaired immune response and increased host mortality.	Dolgachev et al., 2014
Il-17^{-/-} (Interleukin-17) Notes: Non-conditional knock-out	ATCC8045, ATCC43816 C57BL/6	Increased bacterial burden. Reduced immunisation by non-LPS-neutralizing antibody. No effect on Lcn2 production. Increased host mortality.	Ye te al., 2001 Chan et al., 2009 Cohen et al., 2017
Il-17r^{-/-} (Interleukin 17 receptor) Notes: Non-conditional knock-out	ATCC43816 C57BL/6	Increased bacterial burden in the blood. Impaired immune response and decreased Lcn2 production. Increased host mortality.	Chan et al., 2009 Cai et al., 2012
Il-36g^{-/-} (Interleukin-36 gamma) Notes: Non-conditional knock-out	ATCC43816 C57BL/6	Increased bacterial burden in lung and blood. Impaired cytokine production.	Kovach et al., 2017
Mcsf^{op/op} (Macrophage colony-stimulating factor) Also known as: CSF-1 Notes: Mice homozygous for an inactivating mutation in the M-CSF locus (op/op).	ATCC43816 C57BL/6	Increased bacterial burdens. Increased tissue damage and increased host mortality.	Bettina et al., 2016
MglI^{-/-} (Macrophage Galactose-Type Lectin-1) Notes: Non-conditional knock-out.	ATCC43816 C57BL/6	No effect on bacterial burdens. Increased tissue damage and inflammation, increased host mortality.	Jondle wt al., 2016
Mif^{-/-} (Macrophage migration inhibitory factor) Notes: Non-conditional knock-out.	Caroli strain (B5055) BALB/c	Increased bacterial burdens. Increased disease severity and increased host mortality.	Roger et al., 2013
Mirp14^{-/-} (migration inhibitory factor-related protein 14) Also known as: S100A9, calgranulin B. Notes: Non-conditional knock-out. The proteins S100A8 and S100A9 form a heterodimer called calprotectin, a metal-ion-sequestering complex with antimicrobial properties.	ATCC43816 C57BL/6	Increased bacterial dissemination. Increased tissue damage and increased host mortality.	Achouiti et al., 2012
Myd88^{-/-} (Myeloid differentiation primary response 88) Notes: Non-conditional knock-out.	ATCC43816 C57BL/6	Decreased Lcn2 production.	Chan et al., 2009
Nbeal^{-/-} (Neurobeachin-like 2) Notes: Non-conditional knock-out used to model grey platelet syndrome	ATCC43816 C57BL/6	No effect on bacterial burdens. Increased tissue damage and increased leukocyte migration to bronchoalveolar space.	Clashuis et al., 2018
Nlr4/Il-1r^{-/-} (NLR Family CARD Domain Containing 4/Interleukin-1 receptor) Notes: Double non-conditional knock-out.	ATCC43816 C57BL/6	Impaired immune response; impaired neutrophil recruitment not rescued by exogenous IL-1 β administration.	Cai et al., 2012
Nlrp3^{-/-} (NLR Family Pyrin Domain Containing 3) Notes: Non-conditional knock-out.	ATCC43816 Not stated	No effect on bacterial burdens. Decreased airway inflammation and increased host mortality.	Willingham et al., 2009

Table 3.2 Disadvantageous mouse genetic alterations, including mouse and KP strain with outcomes of infection (continued).

Host Genetic Mutation	KP/mouse strain	Effect on <i>Klebsiella pneumoniae</i> infection	Reference(s)
MUTATIONS DISADVANTAGEOUS TO HOST FOLLOWING INFECTION			
ob/ob, Cpe^{fa/fa} Notes: Germ-line ob mutation in the leptin receptor or the carboxypeptidase E gene used as obesity and type 2 diabetes models (<i>Cpe^{fa/fa}</i> mutation mainly causes diabetes in males only).	ATCC43816 C57BL/6	Impaired bacterial clearance at 24 hpi, Increased spleen bacterial counts at 48 hpi. No differences in airspace neutrophil infiltration between lean and obese mice at 24 hpi.	Ubags et al., 2016
Rag2/Il2g^{-/-} (Recombination activating gene 2/Interleukin receptor common gamma chain) Notes: Non-conditional knock-out that results in a lack of B-cells, T-cells and NK cells.	ATCC43816, K. pneumoniae-396 (serotype K2) C57BL/6	Increased lung bacterial burdens in non-immunised mice (not directly compared). Intranasal immunisation with heat-killed bacteria no longer protective.	Chen et al., 2011
Splunc1^{-/-} (Short palate lung and nasal epithelial clone 1) Also known as: SPURT, LUNX, NASG, or BPIFA1 Notes: Non-conditional knock-out of SPLUNC1, a member of the bactericidal/permeability-increasing (BPI) fold-containing (BPIF) protein family.	ATCC43816 C3H	Increased bacterial burden. Increased immune cell infiltration.	Liu et al., 2013
Tlr4^{-/-} or Tlr4^{lps-4} (Toll-like receptor 4) Notes: Non-conditional mutations causing defective TLR4 signalling.	ATCC43816 C57BL/10ScNJ, BALB/c	Impaired lung bacterial clearance. Impaired immune response. Decreased Lcn2 and IFNγ production. Increased host mortality.	Chan et al., 2009 Bhan et al., 2010
Tlr9^{-/-} (Toll-like receptor 9) Also known as: CD289 Notes: Non-conditional knock-out	ATCC43816 BALB/c	Impaired lung bacterial clearance. Impaired immune response and increased host mortality.	Bhan et al., 2010
Tlr4/9^{-/-} (Toll-like receptor 4/Toll-like receptor 9) Notes: Double non-conditional knock-out.	ATCC43816 BALB/c	Impaired lung bacterial clearance (higher compared to single KO). Impaired immune response and increased host mortality.	Bhan et al., 2010
Trif^{-/-} (TIR-domain-containing adapter-inducing interferon- β) Notes: Non-conditional knock-out.	ATCC43816 C57BL/6	Decreased Lcn2 production.	Chan et al., 2009

Table 3.3 Neutral or advantageous mouse genetic alterations, including mouse and KP strain with outcomes of infection.

Host Genetic Mutation	KP/mouse strain	Effect on <i>Klebsiella pneumoniae</i> infection	Reference(s)
MUTATIONS NEUTRAL TO HOST FOLLOWING INFECTION			
C5^{-/-} (Complement component 5) Notes: Non-conditional knock-out. Two-hit model of recurrent, sequential, bacterial aspiration.	ATCC43816 B10.D2	Not required for suppression of growth of second <i>K. pneumoniae</i> inoculum in a two-hit model of recurrent bacterial aspiration.	Ben-David et al., 2005
Il12p35^{-/-}, Il-12/23p40^{-/-} & Il-23p19^{-/-} Notes: Non-conditional knock-out.	ATCC43816 C57BL/6	No effect on Lcn2 production.	Chan et al., 2009
I122^{-/-} (Interleukin 22) Notes: Non-conditional knock-out.	KP35 C57BL/6	No effect on bacterial burdens.	Ahn et al., 2019
Ne^{-/-} (Neutrophil elastase) Notes: Non-conditional knock-out.	Kp52145 129S2	No NET formation.	Papayannopoulos et al., 2010
Sting^{-/-} (Stimulator of Interferon Genes) Also known as: Tmem173 Notes: Non-conditional knock-out.	TOP52 C57BL/6	No effect on <i>K. pneumoniae</i> burden.	Rosen et al., 2018
MUTATIONS ADVANTAGEOUS TO HOST FOLLOWING INFECTION			
A2bR^{-/-} (Adenosine A2b receptor) Notes: Homozygous mice with β-galactosidase knockin at the A2BR locus	ATCC43816 C57BL/6	Decreased bacterial burden in lungs. No difference in leukocyte recruitment to the lungs or expression of inflammatory cytokines, but A2bR^{-/-} neutrophils exhibited six-fold greater bactericidal activity.	Barletta et al., 2012
Chit^{-/-} (Chitinase) Notes: Non-conditional knock-out.	ATCC43816 C57BL/6	Decreased bacterial dissemination. Decreased weight loss, no change in inflammatory responses. Decreased host mortality.	Sharma et al., 2018
Dpp1^{-/-} (Dipeptidyl peptidase I) Notes: Non-conditional knock-out.	ATCC43816 C57BL6	Enhanced bacterial clearance. Increased host survival.	Sutherland et al., 2014
GPx1^{-/-} (Glutathione peroxidase 1) Notes: Non-conditional knock-out.	ATCC43816 C57BL6	Increased transient activation of the innate immune response and decreased host mortality.	Huet et al., 2017
hFER overexpression (non-receptor tyrosine Kinase) Notes: Transient expression by electroporation of plasmid encoded human FER (non-receptor tyrosine kinase) after delivery into the lungs via hypopharyngeal administration.	ATCC43816 C57BL/6	Increased bacterial clearance. Decreased host mortality.	Dolgachev et al., 2018
Hif-1α^{-/-} (Hypoxia-inducible factor 1α) Notes: Transgenic mice that have a doxycycline- inducible lung epithelial cell-specific Hif1α deletion.	ATCC43816 C57BL/6	Decreased siderophore-dependent bacterial dissemination to the spleen after 24 h. No HIF-1α-dependent differences in lung cytokine secretion.	Holden et al., 2016

Table 3.3 Neutral or advantageous mouse genetic alterations, including mouse and KP strain with outcomes of infection (continued).

Host Genetic Mutation	KP/mouse strain	Effect on <i>Klebsiella pneumoniae</i> infection	Reference(s)
MUTATIONS ADVANTAGEOUS TO HOST FOLLOWING INFECTION			
Ifnlr1^{-/-} (Interferon Lambda Receptor 1) Notes: Non-conditional knock-out.	KP35 C57BL/6	Decreased bacterial burden in the lung and blood. Decreased inflammation.	Ahn et al., 2019
Mip-1a overexpression (Macrophage inflammatory protein 1-α) Also known as: CCL3 Notes: transient intrapulmonary overexpression by adenoviral vector	ATCC43816 C57BL/6	Decreased bacterial burden in lung and blood. Increased neutrophil and NK cell recruitment.	Zeng et al., 2003
Nkcc1^{-/-} (Na-K-Cl cotransporter) Notes: Non-conditional knock-out.	ATCC43816 Hybrid C57BL/6 and 129/SvEv	Decreased lung and blood bacterial burdens. Reduced hypothermic sepsis.	Sharma et al., 2018
MUTATIONS WITH VARIABLE/INCONCLUSIVE EFFECTS ON HOST FOLLOWING INFECTION			
Nlr4^{-/-} (NLR Family CARD Domain Containing 4) Notes: Non-conditional knock-out Contradicting observations regarding host mortality and bacterial burdens in two different studies using same strain and IT inoculation	ATCC43816 C57BL/6 (Cai et al., 2012; not described in Willingham et al., 2009)	Increased host bacterial burdens (Cai et al., 2012). No effect on bacterial burden (Willingham et al., 2009). Impaired immune response and increased host mortality (Cai et al., 2012). No effect on host mortality (Willingham et al., 2009).	Willingham et al., 2009 Cai et al., 2012
Ptx3 overexpression (Pentraxin-related protein PTX3) Notes: Different host outcome depending on the bacterial dose. Whole body germ-line mutation.	ATCC27736 CD-1	Greater dissemination of bacteria to blood at 20 h when infecting with high bacterial CFU. Impaired neutrophil recruitment and higher host mortality at high inoculation CFU (3x10⁶ and 3x10⁸). Increased neutrophil recruitment at 2.5h post-infection and increased host survival when infecting with lower inoculation CFU (3x10⁴).	Soares et al., 2006

strengthen this finding, which is biologically plausible given the association with KP infection in alcohol excess in patients. A broad range of other conditions were modelled in mice, all of which were detrimental to the host, except for the case of allergic airway inflammation induced by aerosolized ovalbumin after ovalbumin-alum sensitisation¹⁰⁶. In this case, after KP challenge, reduced bacterial burdens were observed in the lung, spleen and blood which translated into decreased host mortality¹⁰⁶. Neutropaenia was induced in 17 different studies to condition mice to be permissive for infection, especially when clinical KP isolates were used for experimental infection (**Table 3.4**). Neutrophil depletion was achieved by the administration of the chemotherapeutic cyclophosphamide, which can also decrease other leukocyte populations¹⁰⁷, or by the administration of anti-Ly6G monoclonal antibodies. This was the most common conditioning identified of all induced co-morbidities in mice. Lastly, renal impairment was induced by the administration of uranyl nitrate on two occasions. This induces acute renal tubular damage and acute kidney injury in mice and ‘humanises’ renal excretion and drug handling as mice inherently have a much higher renal clearance¹⁰⁸. In agreement with this, these studies were not assessing the role of renal impairment on KP pathogenesis but aimed to simulate human renal physiology and I therefore list them in the second part of **Table 3.4**.

3.2.4. Intervention I: *Klebsiella pneumoniae* strains employed in experimental infections

In the 152 studies, 216 KP strains were used as some studies used more than one KP isolate (**Figure 3.2A**). Five studies did not state what strain had been used. I combined Kp52.145 and B5055 into a single category as Kp52.145 was derived from B5055 and I chose to use the original nomenclature¹⁰⁹. The most commonly used strain for infections was ATCC43816 or its derivatives (33.33%, 72/216) (**Figure 3.2A**) and this was followed by B5055 (9.26%, 20/216). These strains are frequently used as they result in robust infection in the murine host; however, clinical isolates or strains used in <2 studies occurred on 106 occasions. There is less information on the infection kinetics of these isolates, which were most often used in single studies and are generally considered to be less virulent in the mouse. I sought indirect evidence of reduced virulence by assessing if these isolates were passaged through mice, a process thought to adapt them to the murine host. Eight studies mention passaging KP strains prior to infecting mice. In three studies ATCC27736^{96,110,111} was passaged 10 times and in a further three studies ATCC700603 was also passaged 10 times^{112–114}. In one study¹¹⁵, various (8) clinical isolates were sub-cultured on sheep’s blood agar twice prior to infection (i.e. an *ex vivo* passaging approach) and in the remaining study ATCC43816 was routinely passaged in mice¹¹⁶. I was unable to ascertain the role of the passage in host adaptation as, whilst the process was mentioned in methods, no numerical data for passage number and infection outcomes was provided in the studied manuscripts.

Table 3.4 Co-morbidities investigated in mice including conditioning method, mouse and KP strains with outcomes of infection.

Induced co-morbidity (number of studies)	KP/mouse strain	Effect on <i>Klebsiella pneumoniae</i> infection	Reference(s)
CO-MORBIDITIES INDUCED TO INVESTIGATE A HOST CO-MORBIDITY-			
Alcohol consumption (5) Induction method: Ethanol in drinking water/i.p. administration. Rationale: To investigate the association between alcoholism and <i>K. pneumoniae</i> infection	Multiple <i>K. pneumoniae</i> strains Multiple mouse strains	Increased bacterial burden. Increased tissue damage, impaired immune responses and increased host mortality.	Shellito et al., 2001 Spies et al., 2008 Lanzke et al., 2007 Lanzke et al., 2017 Samuelson et al., 2017
Allergic airway inflammation (1) Induction method: Sensitization with ovalbumin-alum i.p. followed by aerosolized ovalbumin challenge before infection. Rationale: To investigate the impact of pre-existing allergic airway inflammation on <i>K. pneumoniae</i> infection.	ATCC43816 BALB/c	Decreased bacterial burden in the lung, spleen and blood. Decreased host mortality.	Dulek et al., 2014
Burn injury (cutaneous) (2) Induction method: Exposure of bare skin to hot water (60°C) or heated brass probes (100°C). Rationale: To investigate the effect of cutaneous burn injuries (± treatment) on <i>K. pneumoniae</i> infection.	ATCC43816 and not stated C57BL/6	Impaired immune responses and increased host mortality.	Horton et al., 2006 Ipakhtchi et al., 2007
Inhaled corticosteroid exposure (1) Induction method: Inhaled fluticasone propionate. Rationale: To investigate the role of ICS treatment in chronic obstructive pulmonary disease (COPD) and <i>K. pneumoniae</i> infection.	ATCC43816 C57BL/6	Increased bacterial burden in lungs and blood. Impaired cytokine expression in alveolar macrophages.	Patterson et al., 2012
Febrile-range hyperthermia (1) Induction method: Core temperature increased from 36.5–37°C to 39.5–40°C by ambient environment heating. Rationale: To investigate the impact of exposure to febrile-range hyperthermia (FRH) in <i>K. pneumoniae</i> infection.	B5055 CD-1	Reduced bacterial burden in the lungs. Increased host mortality, especially in antibiotic-treated mice.	Rice et al., 2005
Immunocompromise (2) Induction method: Sub-lethal irradiation. Rationale: To investigate the role of immunocompromise on <i>K. pneumoniae</i> infection.	AFRRI 7 B6D2F1/J	Irradiation used as a disease model for immunocompromise (therefore, carried out in the absence of non-immunocompromised control group).	Keller et al., 2003 Bentzel et al., 2004
Macrophage colony stimulating factor (M-CSF) depletion (1) Induction method: Anti-MCSF monoclonal antibody i.p. administration. Rationale: To investigate the role of M-CSF in mononuclear phagocyte-mediated host defences in <i>K. pneumoniae</i> infection.	ATCC43816 C57BL/6	Increased bacterial burden in lungs and blood. Increased tissue damage and host mortality.	Bettina et al., 2016
Lung contusion injury (1) Induction method: Blunt (mechanical) lung contusion injury. Rationale: To investigate the role of lung contusion on <i>K. pneumoniae</i> infection.	ATCC43816 FVB/n	Increased bacterial burdens in lungs and blood. Increased host mortality.	Dolgachev et al., 2014
Obesity (1) Induction method: High fat diet (60% (w/w) fat chow for 20 weeks) Rationale: To investigate the role of obesity on <i>K. pneumoniae</i> infection.	ATCC43816 C57BL/6	Impaired bacterial clearance at 48h post infection.	Ubags et al., 2016

Table 3.4 continued

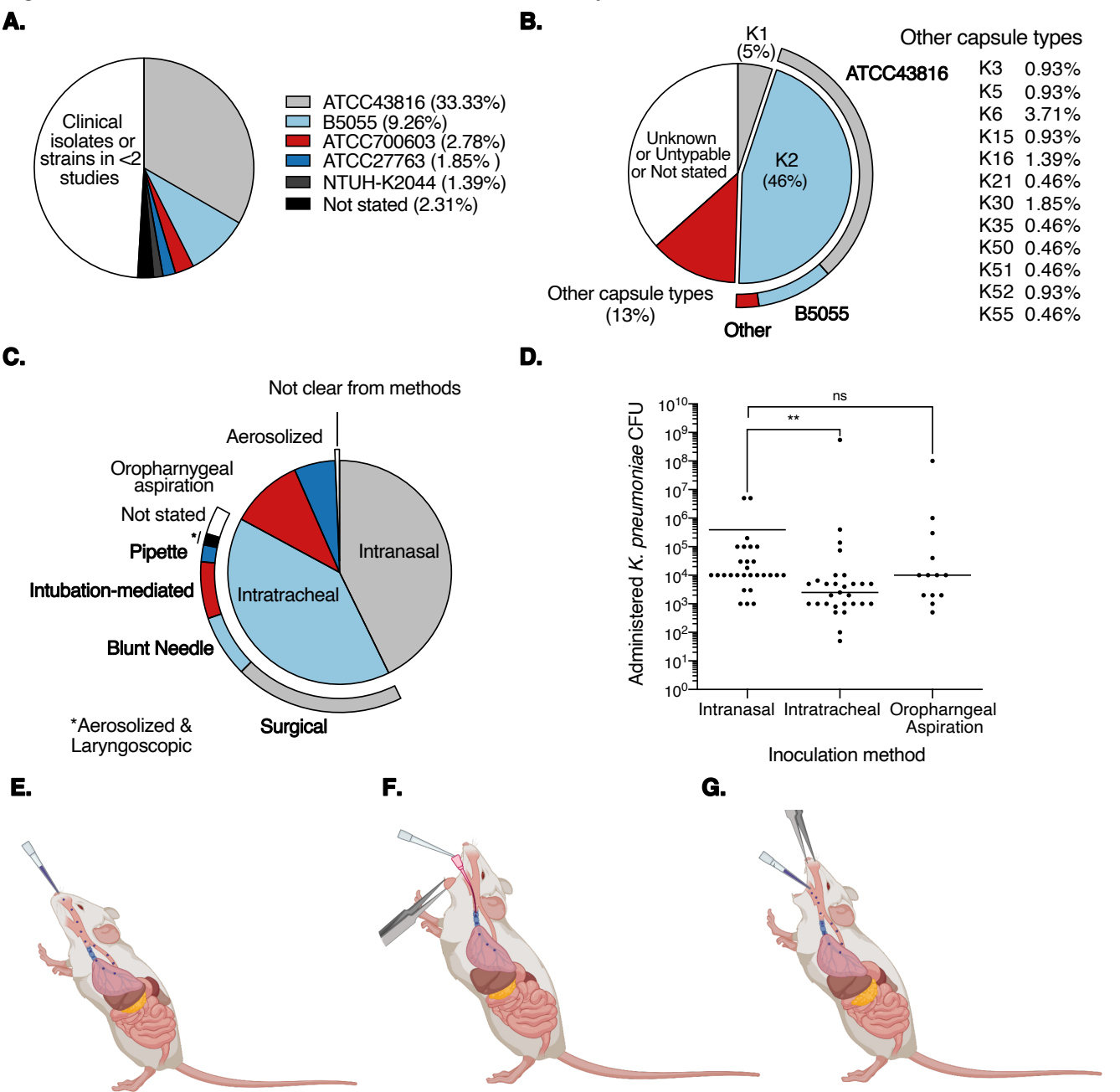
Induced co-morbidity (number of studies)	Effect on <i>Klebsiella pneumoniae</i> infection	Reference(s)
CO-MORBIDITIES INDUCED TO INVESTIGATE A HOST CO-MORBIDITY-		
Thrombocytopenia (1) Induction method: Anti(a)-glycoprotein (Gp)Iba (i.v) administration. Rationale: Investigate the role of Gram-negative pneumonia-derived sepsis induced thrombocytopenia on <i>K. pneumoniae</i> infection.	Increased bacterial burden in lungs, blood, liver and spleen. Increased tissue damage and host mortality.	De Stoppelaar et al., 2014
CO-MORBIDITY INDUCED TO PERMIT INFECTION/SIMULATE HUMAN PHYSIOLOGY		
Neutropaenia (17) Induction method: Cyclophosphamide or anti-Ly6G monoclonal antibody i.p. administration Rationale: To increase susceptibility the <i>K. pneumoniae</i> infection by neutrophil depletion.	Increased lung bacterial burden. Neutropaenia induced to permit infection (therefore, carried out in the absence of non-neutropaenic control group).	Mine et al., 1993 Andes et al., 2002 Kostina et al., 2005 Zuluaga et al., 2006 Craig et al., 2008 Barietta et al., 2012 Agudelo et al., 2013 Hirsch et al., 2013 Agudelo et al., 2014 Thabit et al., 2016 Lepak et al., 2016 Landersdorfer et al., 2017 Monogue et al., 2017 Sabet et al., 2017 Zhou et al., 2017 Geiler et al., 2018 Asempa et al., 2019
Renal impairment (2) Induction method: Uranyl nitrate (i.p) administration. Notes: Renal failure induced to reduce renal clearance enabling 'humanised' antibiotic pharmacokinetic profiles/exposures.	Renal failure induced to 'humanise' murine renal function (therefore, carried out in the absence of control group without renal impairment).	Hirsch et al., 2013; Asempa et al., 2019

Capsule (extracellular polysaccharide) and capsule type (composition) are regarded as a key virulence factor in KP pathogenesis, I specifically looked for this data in the manuscript collection. In over half of the studies K1 and K2 capsule type KP strains were used (**Figure 3.2B**). In many cases no associated capsule serotype or genotype data for strains were available. The large proportion of K2 strains used in experimental infection is directly attributable to the use of ATC43816 and B5055, both of which encode K2 capsule loci. I wanted to examine the relationship between capsule types used in mouse infections and circulating capsule types in contemporary clinical isolates. To that end, I used published data from a large collection of genetically sequenced European KP isolates collected as part of a structured antimicrobial resistance survey (EuSCAPE)²⁴. I found that only 4.6% (79/1717) of strains infecting or colonising human patients (collected in 2013-2014) belonged to either K1 or K2 capsule types

3.2.5. Intervention II: Comparative analysis of *Klebsiella pneumoniae* inoculation methods

Each study used a single method of inoculation with intranasal (IN) and intratracheal (IT) routes being encountered most commonly (**Figure 3.2C**). Oropharyngeal aspiration and inoculum aerosolization represented alternative, less commonly used methods. IT delivery was achieved by several methods including surgical tracheotomy, a blunt needle technique (blind) or an intubation-mediated approach, in which a temporary catheter is inserted into the trachea under direct visualisation of the mouse vocal cords. Whilst the methodology of choice is related to the skillset within the laboratories conducting the work, I hypothesized that inoculation technique dictates the dosage of bacteria administered to mice. To that end, I analysed studies that infected mice with ATCC43816 (and its derivatives) and compared the dose of bacteria administered by route of administration (**Figure 3.2D**). I used a single strain for this analysis to remove bias resulting from the intrinsic capacity for murine infection by different strains and chose ATCC43186 as it was utilised most frequently, thus providing a larger dataset. This analysis demonstrated that the inoculation route does determine the dose of bacteria used to generate experimental infection with significantly lower doses of ATCC43816 used in studies when administered IT rather than IN (**Figure 3.2D**). The IT route ensures direct access to the trachea and delivery of the complete inoculum into the lungs, therefore avoiding the soiling of the upper airways that occurs during IN delivery and the inadvertent gastrointestinal tract administration via the oesophagus, which will occur in both IN and oropharyngeal aspiration methods. These differences may underpin the different KP doses used in these methods. The anatomical features of these delivery methods are shown in **Figures 3.2E-G**, including the potential routes that inoculum can take in each case. \

Figure 3.2 Features of KP strains and methods used for experimental infection.



A. 216 different KP strains were administered to mice; in 5 studies no information on KP strain was provided. ATCC43816 (and its derivatives) was the most used KP strain followed by B5055. Clinical isolates or strains used in ≤ 2 studies were combined into a single category for the purpose of this analysis. **B.** Most studies (64%) provided information on capsule type (K serotype or K locus). Together, over half of studies use K1 and K2 capsule expressing strains. The predominant use of K2 capsule types is directly attributable to the use of ATCC43816 and B5055 as shown in (A). The outer ring illustrates the proportion of K2 infections by strain. **C.** Inoculation methods used to administer KP into the lungs. Intranasal and intratracheal methods were used most frequently. The intratracheal route is further broken down (outer ring) as multiple different techniques were employed. **D.** An analysis of the dose of ATCC43816 (median dose used if range provided) by either intranasal (IN, $n=25$), intratracheal (IT, $n=26$) or oropharyngeal aspiration (OA, $n=12$). Significantly less inoculum was delivered by the IT route compared to IN route to achieve infection. Analysis by ordinary one-way ANOVA and Tukey's post-test for multiple comparisons between groups. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$. **E-G.** Cartoon depictions of IN (E), IT (intubation-mediated, F) and OA (G) administration techniques. Diagrams demonstrate the path inoculum take (blue dots) into the lungs. In IN (E) and OA (G) methods some inoculum may pass into the oesophagus via the shared upper airway. The IT route is infraglottic and inoculum avoids oesophageal soiling (F).

3.2.6. Comparisons: *Klebsiella pneumoniae* mutants evaluated in experimental infections.

I identified 27 studies that used mutant KP strain for infections in mice (Table 3.5). Two studies used spontaneous unencapsulated mutants and, as the genetic origin of the phenotype was not known, I did not include them in this analysis^{117,118}. One study used a transposon library to determine the roles of multiple genes in lung infection and, due to the large number of genes mutated, this was also not included in the data summary³⁰. In addition, genetic manipulation was used to tag strains for *in vivo* imaging purposes (2 occurrences) and to introduce *qnrA1* to confer fluoroquinolone resistance (1 occurrence)^{119–121}. These were not introduced for the purpose of studying the effect of these genes on KP infection and thus were also excluded from our summary table (Table 3.5). All the studies listed targeted specific genes and reported the impact the disruption relative to an isogenic wild-type (WT) KP infection. Virulence in these studies focused on the bacterial burdens achieved in the lung tissue and/or host survival. Most mutations were introduced into genes encoding for virulence factors and, given the major role attributed to capsule polysaccharide and lipopolysaccharide, genes involved in this pathway were the most commonly investigated (Table 3.5). Despite the key proposed role of capsule in KP pathogenesis, not all mutations resulted in attenuation following lung infection, although this was the most typical effect. In the case of the O-antigen ligase, *waaL*, groups came to opposing conclusions (despite both using the same B5055 KP strain background) with disruption in one case proposed to be neutral and in one case deleterious^{122,123}. Other key virulence factors involved in siderophore synthesis and uptake or type 1 and type 3 fimbriae synthesis or structural subunits similarly demonstrated mixed results. In several studies of siderophore mutants lung burdens were not different as compared to WT strains but a failure to disseminate was demonstrated by reduced KP recovery from the spleen¹²⁴. In agreement with the targeting of virulence factors, no mutations were shown to lead to increased bacterial burdens.

3.2.7. Outcomes I: Bacterial burden and host inflammatory response assessments.

Colony forming units (CFU) counts were used as a measure of infection outcome in 84.8% (129/152) of studies and data were collected at specified time points post infection in 96.9% (125/129) of these manuscripts. I was unable to identify the time point of tissue collection in four studies and in one study an alternative approach was taken - blood was taken and bacteria enumerated when a predetermined clinical severity score was reached¹²⁵. 58 studies only took samples for CFU counts at a single time point and 62 studies only enumerated bacteria from a single site, which was mostly represented by lung tissue. In general, most studies tended to measure CFU counts after a minimum of 24 h.

In order to summarise these data, I generated a chart that breaks down infections by the strain used to infect the mice and stratifies CFU enumeration by the site of infection and the time at which sampling took

place (**Figure 3.3**). This highlighted the common time periods of assessment, which most frequently lay in the 24-48 hour or >48 hours period. This sampling kinetic appears to be independent of the KP strain used for experimental infection. Together with whole lungs, that were usually homogenised during processing, blood, spleen, liver and bronchoalveolar fluid (BALF) were obtained and used for bacterial burden enumeration by researchers in decreasing frequency. It is plausible that lung and BALF represent similar metrics although BALF likely only represents intra-alveolar bacterial numbers. In the case of blood, spleen and liver, these are markers of dissemination, where bacterial counts in blood also depend on the ability of the bacteria to survive complement attack¹²⁶. I observed no discernible differences between the infecting strain and site of collection (**Figure 3.3**).

38% (58/152) of studies performed an analysis of cytokines and/or chemokines to assess the mouse immune response to KP infection. Similarly to CFU enumeration, these were measured from different sites/sample types and at different time-points after infection between studies. In one case I was unable to identify the time point of collection and excluded this from our analysis¹²⁵. I summarised these data into a chart (**Figure 3.4**) and stratified the assessed cytokines/chemokines by sample type and time post infection. In total 36 different cytokines or chemokines were measured, and the most common sample type was lung homogenate (57%) followed by BALF (47%) and serum/plasma (24%). While studies typically assessed cytokines and/or chemokines from a single source, 14 studies measured levels at a combination of two sample types and one study obtained and reported measurements from serum, BALF and lung homogenate samples¹²⁷. While TNF and IL-6 were the most common analytes, IL-1 β and CXCL1 (Kc; 1L-8 in humans) and CXCL2 were also frequently measured. The alarmins HMGB1 and IL-1 α , whose release is also associated with inflammasome activation¹²⁸, were less frequently quantified (**Figure 3.4**). While also not technically a cytokine, IL-1RA was included in this chart as it has a direct impact on the response to cytokines of the IL-1 family and its recombinant form is commercially available as Anakinra¹²⁹. IFN γ was the most frequently assessed member of the interferon family. In line with the CFU outcome data (**Figure 3.3**), host immune responses were typically measured in the 24-48 and >48 h period, presumably as samples were collected for both measurements simultaneously, at a time point when mice were sacrificed. The values obtained for pro-inflammatory cytokines and chemokines usually reflected similar patterns regardless of the type of sample in which they were measured, where these immune markers increased in abundance as the infection progressed until the 48-72h endpoint. Infections lasting for longer periods were rare and usually reflected infection recovery models¹³⁰. Interestingly, one cytokine that was also investigated on multiple occasions was IL-10 (also known as human cytokine synthesis inhibitory factor, CSIF) which, in contrast, is an anti-inflammatory immunomodulatory factor¹³¹.

Table 3.5 KP mutants investigated including parental KP strain, the outcome of infection and the outcome measure assessed.

Mutated gene/s and encoded protein (number of studies)	Parental K. pneumoniae strain	Outcome of lung infection (number of studies)	Outcome Measure	Reference(s)
CAPSULE AND LPS SYNTHESIS				
<i>cpsB/manC</i> Mannose-1-phosphate guanylyltransferase (3)	ATCC43816 (2), Kp8570	Attenuated (3)	Lung CFU	Lawlor et al., 2006 Lawlor et al., 2005 Jones-Nelson et al., 2018
<i>lpxM</i> Lipid A biosynthesis myristoyltransferase	B5055	Attenuated	Lung CFU	Clements et al., 2007
<i>waaQ</i> Lipopolysaccharide heptosyltransferase III (2)	B5055 (2)	Attenuated (2)	Lung CFU; Survival	Fresno et al., 2006 Benghezal et al., 2005
<i>wabG</i> GalA transferase	B5055	Attenuated	Lung CFU	Fresno et al., 2006
<i>wabO</i> Hypothetical GalA transferase	B5055	Attenuated	Lung CFU	Fresno et al., 2006
<i>wbbM</i> Putative glycosyltransferase	B5055	Attenuated	Survival	Benghezal et al., 2005
<i>rfbB</i> O-antigen export system ATP-binding protein RfbB	ATCC43816	Attenuated	Lung CFU	Lawlor et al., 2005
<i>waaL</i> O-antigen ligase family protein (2)	B5055 (2)	Neutral (1), Attenuated (1)	Lung CFU	Fresno et al., 2006 Tomías et al., 2015
<i>galK</i> Galactokinase	ATCC43816	Neutral	Lung CFU	Ben-David et al., 2005
<i>nagA</i> N-acetylglucosamine-6-phosphate deacetylase	B5055	Neutral	Survival	Benghezal et al., 2005
<i>wecA</i> Undecaprenyl-phosphate alpha-N-acetylglucosaminyl 1-phosphate transferase	ATCC43816	Neutral but less able to disseminate	Lung CFU	Lawlor et al., 2005
<i>wecE</i> dTDP-4-amino-4,6-dideoxygalactose transaminase	ATCC43816	Neutral	Lung CFU	Lawlor et al., 2005
<i>wecG</i> UDP-N-acetyl-D-mannosaminuronic acid transferase	ATCC43816	Neutral	Lung CFU	Lawlor et al., 2005
<i>wzyE</i> Hypothetical ECA synthase	ATCC43816	Neutral	Lung CFU	Lawlor et al., 2005
SIDEROPHORES SYNTHESIS AND UPTAKE				
<i>ybtS</i> Yersiniabactin biosynthesis salicylate synthase (3)	ATCC43816 (3)	Neutral* (1), Attenuated (2)	Lung CFU	Holden et al., 2016* Bachman et al., 2011 Lawlor et al., 2007
<i>tonB</i> Protein TonB	ATCC43816	Neutral	Lung CFU	Holden et al., 2016
<i>entB</i> (3) Enterobactin synthase component B	ATCC43816 (3)	Neutral* (3)	Lung CFU	Holden et al., 2016, Bachman et al., 2011 Lawlor et al., 2007
<i>gly-ent</i> (2) Glycosylase (iroB)	ATCC43816 (2)	Neutral* (2)	Lung CFU	Bachman et al., 2011 Holden et al., 2016
FIMBRIAL GENES				
Type 1 pilus operon <i>fimS</i> - <i>fimK</i>	TOP52	Attenuated (assumed)	Lung CFU	Rosen et al., 2017
<i>fimK</i> Fimbrial adhesin (2)	C3091, TOP52	Neutral (2)	Lung CFU	Struve et al., 2009
<i>mriA</i> (2) Fimbrial subunit type 3	TOP52, C3091	Neutral (2)	Lung CFU	Rosen et al., 2017 Struve et al., 2009
METABOLISM				
<i>pld</i> Phospholipase D	B5055	Attenuated	Survival	Lery et al., 2014
<i>peg-344</i> Hypothesized metabolite transporter	hvKp1	Attenuated	Lung CFU	Bulger et al., 2017
<i>leuABCD</i> Leucine synthesis gene cluster	ATCC43816	Attenuated	Lung CFU	Wilsey et al., 2018
<i>proV</i> Glycine betaine/L-proline ABC transporter ATP-binding protein	ATCC43816	Attenuated	Lung CFU	Wilsey et al., 2018
<i>metB</i> Cystathionine gamma-synthase	B5055	Attenuated	Survival	Benghezal et al., 2005

Table 3.5 continued

Mutated gene/s and encoded protein (number of studies)	Parental <i>K. pneumoniae</i> strain	Outcome of lung infection (number of studies)	Outcome Measure	Reference(s)
METABOLISM				
<i>trpC</i> Multifunctional fusion protein	B5055	Attenuated	Survival	Benghezal et al., 2005
ANTIMICROBIAL RESISTANCE				
<i>acrB</i> Efflux pump membrane transporter	B5055	Attenuated	Lung CFU	Padilla et al., 2009
<i>mdtJ</i> Spermidine export protein MdtJ	ATCC43816	Attenuated	Lung CFU	Willsey et al., 2018
OTHER PROCESSES				
<i>pilA</i> Pullulanase	B5055	Attenuated	Lung CFU	Tomás et al., 2015
<i>qrgB</i> from <i>Vibrio harveyi</i> / Diguanylate cyclase **	TOP52	Attenuated	Lung CFU	Rosen et al., 2017
<i>ompA</i> Outer membrane protein A	B5055	Attenuated	Lung CFU	March et al., 2011
<i>Dam</i> Site-specific DNA-methyltransferase (adenine-specific)	B5055	Attenuated	Survival	Benghezal et al., 2005
<i>kvrA</i> MarR family protein	ATCC43816, NTUH-K2044, MKP103	Attenuated	Lung CFU	Palacios et al., 2018
<i>kvrB</i> MarR family protein	ATCC43816, NTUH-K2044, MKP103	Attenuated	Lung CFU	Palacios et al., 2018

* Attenuated in ability to disseminate to spleen, this was also at an earlier time point compared to the other two studies (24h vs 72h)
**non-Klebsiella gene with inducible expression off a plasmid

3.2.8. Outcomes II: Physiological parameters employed in assessing experimental infection

25/152 studies measured physiological parameters in mice experimentally administered KP into the lungs; these parameters were either measured at endpoint or dynamically during the acute or recovery phases of the infection; the results are summarised in **Table 3.6**. In some cases, the data were not included in the manuscript but described in the materials and methods. This was frequently the case when a severity scoring system was used to determine experimental endpoint, instead of time post infection. In some studies, more than one parameter was measured (e.g. body weight and severity scoring system^{132,133}). I found that body weight was the most frequently reported physiological outcome of infection (n=10). This is usually calculated as the difference between starting (pre-infection) and finishing weight and, in most cases, presented as a percentage of the initial weight to account for heterogeneity in the initial values. Whilst weight loss was demonstrated as a marker of infection, this feature is not specific to KP and is encountered and reported in other murine infection models, for example following oral gavage of *Citrobacter rodentium* into C3H/HeN mice^{134,135}. Clinical scoring systems (n=7), which assess illness severity, were used for this purpose as well as to define severity endpoints for infections and are based on the subjective assessment of clinical signs. Commonly assessed signs include the quality of the fur (i.e. ruffling of fur), posture (i.e. hunching) and movement (i.e. responsiveness to external stimuli). The systemic effects of sepsis on haemodynamics and hypoperfusion with resultant hypotension and increased blood lactate was measured in 4 studies (**Table 3.6**). In a similar fashion the hypothermic systemic response in mice to sepsis, also observed in humans¹³⁶, was also used as a physiological outcome measure in six studies by assessing body temperature. Despite our focus on lung infection the direct effect on pulmonary function and lung mechanics was only investigated in 2 studies (**Table 3.6**), probably due to the difficulties associated in obtaining these measurements. Renal failure (n=1) and liver dysfunction (n=3) were measured by assessing the serum levels of the biomarkers creatinine and alanine and aspartate transaminase (ALT/AST), respectively, which is also done in patients with sepsis and septic shock routinely in clinical practice. Lung histology was carried out post-mortem in 35.3 % (54/152) of studies to provide an insight into the architecture of lung damage induced by KP.

3.2.9. Validation of an intubation-mediated intratracheal inoculation methodology

I proceeded to generate an in-house preclinical KP pneumonia model based on our findings in the published literature. I chose to use ATCC43816 as our model KP strain for infections as this had been widely used in mouse infection models (**Figure 3.2A**) and is genetically tractable (**Table 3.5**). I observed in the literature review that the lung infections with ATCC43816 resulted in pneumonia with dissemination into blood with bacteraemia. This severe infection phenotype was in line with our goal of replicating the

Figure 3.3 Sites and times post-infection that bacterial burdens were assessed as outcome variables.

Strain	t < 24 hours	24 ≤ t < 48 hours	t ≥ 48 hours	Unclear
ATCC43816	(16 26 32 56 71 83 103 108 109 113 143 142 63 97 101 113 143 142 82 53 85 101 107 143 142 63 101 107 113 143 97 146 142 32 107)	12 16 19 20 25 26 29 32 42 44 48 50 54 56 64 71 74 77 78 83 87 91 93 98 101 106 107 109 111 113 114 118 135 139 140 141 142 145 12 19 20 25 44 45 46 50 64 68 83 87 101 113 135 140 141 142 32 42 56 72 78 83 85 101 107 113 143 119 143 145 19 83 101 107 113 142 22 87 87 146 142 32 107)	8 12 16 19 20 25 29 30 32 39 42 47 48 56 59 60 64 71 72 78 79 80 81 83 87 98 113 114 115 118 123 129 135 140 141 149 147 148 152 8 12 19 20 25 47 48 64 79 80 81 85 87 113 115 123 135 143 141 42 45 56 59 62 72 78 79 83 88 113 119 146 145 147 159 59 60 83 113 115 87 146 147 152)	(60 69 103 65 66)
	(43 83 43)	46 62 69 119 27 49 53 57 63 99 105 43 119 43 111 89 119)		
	(85 86 85 85 86 85)	124 85 86 149 85 85 86 85	85 148 85 85 85	
	(38 38)	126 139 139		
		145 43	(13 145 43 51)	(60 60 65 66 6 24 75 24 75)
Clinical isolates and strains in ≤ 2 studies	1 2 3 10 11 35 67 70 112 127 70 112 112 112	2 3 10 35 44 61 65 67 70 77 84 88 92 94 104 108 112 113 120 121 124 125 127 128 130 132 133 134 136 137 145 146 150 151 44 70 94 112 128 136 136 77 119 123 136 43 45 46 124 154 155 155 130)	3 4 10 23 31 33 37 51 52 80 61 67 70 84 94 112 127 130 131 144 146 147 150 70 94 112 133 62 60 112 139 146 147 52 80 63 147 151 55 55 130)	
		(138 56 57 58 59 138)	66	
Not stated				

Colony forming units (CFU) at experimental endpoint were commonly assessed as an outcome variable of infection. Data is summarised in this figure and is broken down by the infecting strain, time point post-infection of collection (<24 hours, 24–48 hours, >48 hours or unclear) and the site at which CFU were measured (indicated by the colour of each circle). The key refers to the site of collection and each circle contains a number referring to the source manuscript (List of numbered manuscripts available in **Appendix 1**).

Figure 3.4 Sites and times post infection that immune responses (cytokines and chemokines) were assessed as outcome variables.

	t < 24 hours	24 ≤ t < 48 hours	t ≥ 48 hours
CCL2	(80 112 142 112 112 112)	44 112 142 138 141 142 63 142	118 141
CCL3	(8 80 112)	8 98 112 87	8 98 87
CCL4	(80)	98	98
CCL5	(112)	112 87	87
CCL8		98	98
CCL20			98 118
CXCL1	(38 64 71 80 83 106 112 117 56 83 106 116 63)	44 64 71 78 81 83 88 106 112 127 136 56 78 83 87 91 98 106 136 83	71 78 81 83 113 118 127 56 78 83 87 141 146 147 83
CXCL2	(8 64 71 80 83 108 16 56 83 86 106 83 88)	8 44 48 64 71 78 81 83 108 16 56 78 83 83 108 83	8 48 71 78 81 83 118 56 78 83 88
CXCL5	(80)	78 78	78 78
CXCL6			113
CXCL9	(25)		
CXCL10	(25 80)	48 141	48 141
G-CSF	(108 16)	98 112 16 91	98
GM-CSF	(108)	98 112	98 113 127
HMGB1		141	141
IFNβ			127
IFNγ	(25 56 64 67 112 98 67)	48 84 87 112 119 121 56 136 141 67 119 136 138	48 113 119 127 128 56 125 140 141 119 138
IFNα			147
IL-1α			128 87 88
IL-1β	(71 83 108 112 127 83 85 106 107 83 85)	71 78 83 98 106 108 112 119 121 127 78 83 85 106 107 83 85 119 138	71 78 83 98 105 113 118 119 127 47 59 78 83 85 140 147 149 59 83 85 88 119
IL-RA			88 141
IL-2		141	140
IL-4		98 121 136 141 88	98 135 140 141 88
IL-5		98 121	71 98 140
IL-6	(67 71 80 83 106 112 127 142 56 83 106 107 142 146 67 83 109 142 106 107 136 142 46 22 67 83 84 101 119 136 42 8 87 83 112 127 83 88)	50 67 71 83 98 106 108 112 119 121 127 136 136 142 22 56 83 87 91 83 106 107 136 142 46 22 67 83 84 101 119 136 42 8 50 87 81 88 106 112 119 128 136 83 136 67 83 119 136 83 48 64 121	33 63 98 113 118 119 127 128 135 47 56 59 83 87 140 146 89 83 88 119 138 8 81 88 105 113 119 127 128 136 47 83 140 83 88 119 138 48 127 123 140
IL-10			98 141
IL-12	(25 64)	98 121 141	78 81 98 127 135 78 78 140 147 138
IL-13		64 78 81 98 112 135 16 78 138 135 139	98 147
IL-17	(64 112 25 64)	98 127	127 123
IL-22	(64)	64	127
IL-23		138 138	
IL-27		115	119
IL-34			123
IL-36			98
M-CSF		115	8 39 69 71 78 83 98 99 105 113 127 128 135 47 56 78 79 83 85 87 140 141 146 147 149 83 85 88 138
TNFα	(8 25 38 39 64 71 80 83 112 127 142 56 83 85 107 142 146 88 83 85 88 109 142 8 83 85 88 109 142)	8 39 63 64 71 78 83 98 99 105 106 112 121 126 127 135 136 142 67 91 107 138 138 141 142 146 149 85 101 83 138 142 8 39 69 71 78 83 98 99 105 113 127 128 135 47 56 78 79 83 85 87 140 141 146 147 149 83 85 88 138	8 39 69 71 78 83 98 99 105 113 127 128 135 47 56 78 79 83 85 87 140 141 146 147 149 83 85 88 138

36 different cytokines or chemokines were measured at experimental end-point as outcome variables of infection. The data is summarised in this figure and broken down by the inflammatory mediator measured, time point post-infection of collection (<24 hours, 24–48 hours, >48 hours) and the sample type in which they were measured. The key refers to the site of collection and each circle contains a number referring to source manuscript (List of numbered manuscripts available in **Appendix 1**).

high morbidity and mortality for critically unwell patients with KP infections⁸². In previous work a spontaneous rifampicin (Rif) resistant mutant of ATCC43816 (achieved by serial passage on rif up to 100mcg/ml) was generated to facilitate antibiotic selection (**Clements, unpublished**). This ATCC43816 Rif^R strain was then passaged twice *in vivo* through female BALB/c mice by IN inoculation. A colony isolated from the lungs in the first passage was employed as the inoculum in the second passage. After the second passage a single colony was saved and was used for all future experiments, and I named this strain ICC8001. ICC8001 is the wild-type (WT) strain employed universally throughout this thesis and it will therefore refer to it as KP_{WT} in *in vivo* and *in vitro* work. A list of strains used in this chapter, with attributes, is listed in **Table 3.7**.

The legal framework required to enable the generation of a severe mouse infection model required significant in-depth discussions with both institutional and Home Office oversight teams. All work needed to comply with the Animals (Scientific Procedures) Act (ASPA) 1986 and it represented the first severe bacterial infection and sepsis model to be approved locally. We put in place several safeguards to ensure animal welfare, including the provision for overnight animal monitoring, enhanced reporting to the Home Office and a communications structure to ensure a member of staff was always available to animal technicians. All work complied with local and national guidance and was approved by our local animal welfare ethical review body (AWERB).

I chose to use 8-10 week old, 18-20g, female BALB/c mice for infections as they had been widely used in the published literature (**Figure 3.1D**) and the serial passage, generating KP_{WT}, had taken place in this breed. I had established that IT inoculation consistently required lower doses of ATCC43816 (**Figure 3.2D**) to establish infection in mice and I therefore adopted this approach in our model. Whilst technically more challenging, I decided that an intubation-mediated approach (c.f. surgical tracheotomy) best replicated the mode of KP acquisition in intensive care, where pulmonary infection frequently occurs in intubated and ventilated patients.

To that end, I initially evaluated the accuracy that I could achieve by intubation-mediated IT administration in female BALB/c mice. KP_{WT} was grown in rich media (LB) to a stationary phase overnight culture and diluted in phosphate-buffered saline (PBS) to achieve an inoculum dose of 1000 CFU in 50ul. Mice were anaesthetised using a combination of ketamine (80mg/kg) and medetomidine (0.8mg/kg) by intraperitoneal (IP) injection. Vertical suspension of animals was achieved by using a commercially available intubation platform and intubation of the trachea took place by passing an endotracheal tube (21G cannula) over an illuminated fibre-optic cable under direct vision of the vocal cord. The cannula was left in place and positioning was confirmed by insufflation of 400ul of air with external chest rise confirming tracheal placement. 10 mice were inoculated with 1000 CFU of KP_{WT} and the inoculum was flushed into

Table 3.6 Physiological metrics assessed as outcome measures of infection.

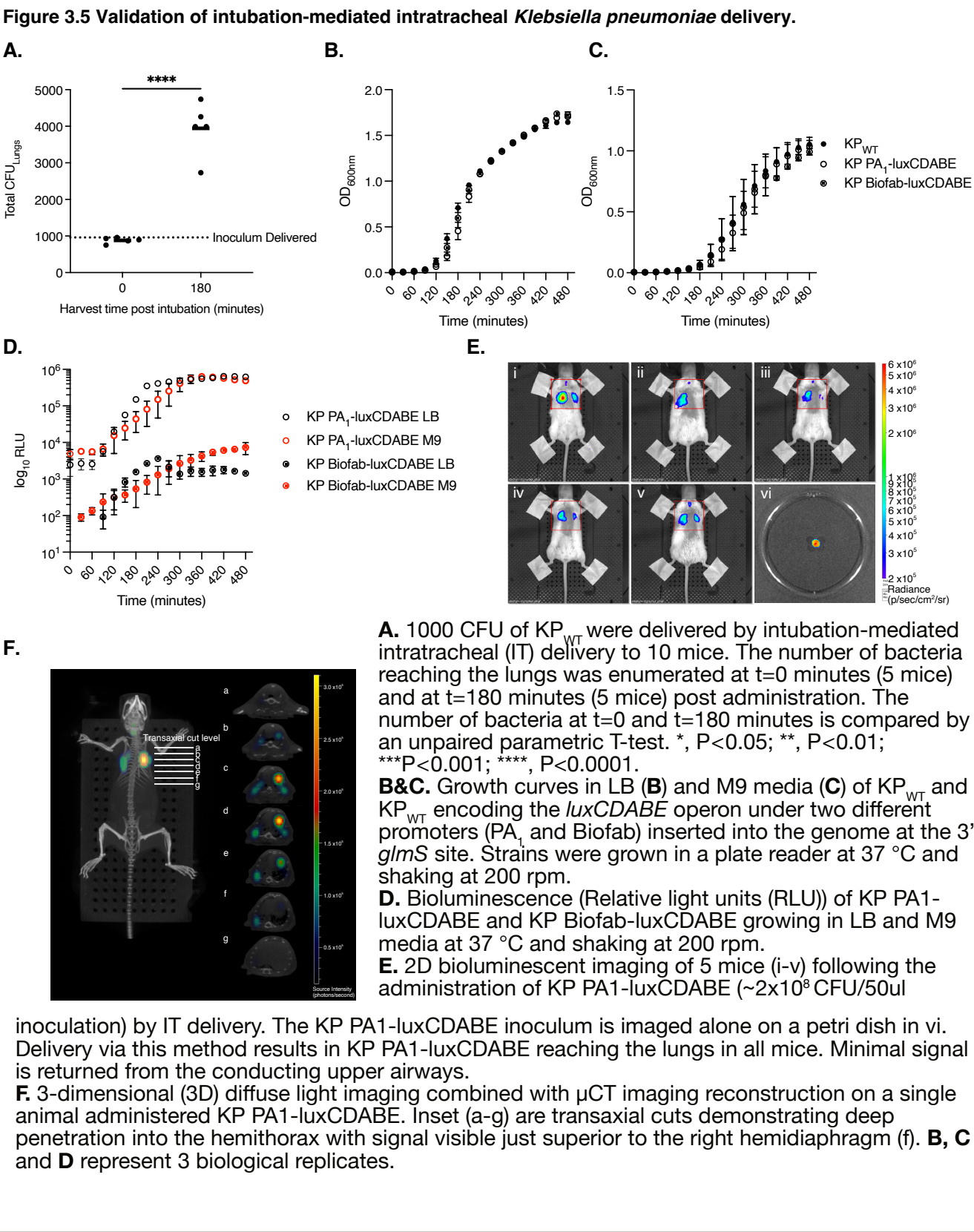
Physiological metric assessed	Details and methodology	Reference(s)
Body weight	Weight loss induced by infection. Calculated as the difference between the pre and post infection weight.	Cao et al., 2015; Kort et al., 1998; Lanzke et al., 2007; Rodgers et al., 2019; Sharma et al., 2018; Spies et al., 2008
Severity scoring system	Observational scoring systems based on clinical signs. These usually assess several metrics (e.g. quality of fur coat, posture, movement) to provide a cumulative score indicating illness severity.	Bachman et al., 2011; Huet et al., 2017, 2013; Lanzke et al., 2017; Roger et al., 2013
Temperature	Rectal thermometry or surgically implanted thermometers have been used in different studies. A hypothermic response in mice is associated with increased illness severity.	Hilliard et al 2011 Kort et al 1998 Nguyen et al 2007 Geller et al 2018
Renal function	Assessment of renal clearance measured by serum creatinine levels. An increase in levels is reflective of reduced renal clearance and acute kidney injury.	
Liver function	Assessment of serum levels of the hepatic enzymes aspartate transaminase (AST) and alanine transaminase (ALT) as markers of liver dysfunction.	
Respiratory mechanics	Respiratory rate, lung volumes and pulmonary compliance measured by whole body plethysmography or ventilator-derived mechanical studies.	Czaikoski et al., 2013 Ipaktchi et al., 2007
Haemodynamics	Arterial blood pressure measurement and vasopressor responses or plasma lactate as a measurement of hypoperfusion in sepsis. Measured by transduction of indwelling arterial catheter placement of lactate measurement in blood.	Bettina et al., 2016 Horewicz et al 2015 Horton et al 2006 Sordi et al 2013
Exercise capacity	Physical capacity tested by maximal distance walked on a miniaturised mouse treadmill	Probst et al 2019

the lungs with 2 x 400ul air flushes to disperse the inoculum suspension into the lungs. I confirmed the accuracy of delivery by enumerating KP_{WT} CFU from lungs from 5 mice immediately after inoculation (**Figure 3.5A**). I confirmed that the number of CFU in the inoculum was accurate by plating a sample (aim 1000 CFU/50ul, actual 960 CFU/50ul) and *a priori* defined inoculum as valid, for all *in vivo* work, if it fell within ±10% of our intended dose. In comparing the CFU of KP_{WT} administered to those recovered I found that this method was highly accurate with 91.9% (SD±8.4%) of the dose reaching the lungs of mice. I allowed the other 5 mice to recover from anaesthesia and pharmacologically reversed the medetomidine anaesthesia with the administration of atipamezole (1mg/kg) by subcutaneous (SC) injection. At 180 minutes post infection I enumerated KP_{WT} from the lungs from these mice. I observed a significant increase in bacterial burdens at this early time point, indicating rapid adaptation and growth of KP_{WT} in the *in vivo* environment (**Figure 3.5A**).

3.2.10. Inoculum localisation and confirmation of lung parenchymal delivery *in vivo* imaging
Having confirmed the dosing accuracy of KP_{WT} I assessed the pulmonary distribution that this method of inoculation achieved. Given I had harvested mouse lungs *en bloc* (i.e. including the trachea and main bronchi) I was unable to ascertain if the bacteria had reached the lower airways or remained largely in the

Table 3.7 <i>Klebsiella pneumoniae</i> strains used in this chapter.		
Isogenic strains		
Strain name	glmS insertion	Notes
KP _{WT}	None	ATCC43816 derivative ICC8001
KP PA ₁ -luxCDABE	PA1-luxCDABE	luxCDABE expression driven by the PA1 phage promoter
KP Biofab-luxCDABE	Biofab-luxCDABE	luxCDABE expression driven by a synthetic Biofab promoter
KP GFP	sfGFP	sfGFP expression driven by a synthetic Biofab promoter
KP RFP	mRFP1	mRFP1 expression driven by a synthetic Biofab promoter
Clinical isolates		
Strain name		Notes
KP _{ST258}		Public Health England KP ST258 isolate expressing the KPC-2 carbapenemase
KP _{OXA-48}		Public Health England KP isolates (of unknown ST) expression the OXA-48 carbapenemase

upper conducting tracts. I therefore used *in vivo* imaging to assess the distribution that IT inoculation achieved. I used a derivative of KP_{WT} that had been genomically-tagged with the bacterial luciferase (*lux*) operon (*luxCDABE*) from *Photorhabdus luminescens*. *luxCDABE* expression results in spontaneous light emission by encoding the enzymes required for substrate generation and photon generation in an ATP-dependent fashion. The KP_{WT} genome had the bacterial *lux* operon inserted into the 3' region downstream of the *glmS* open reading frame (ORF)⁶⁶ as this genomic insertion site has previously been demonstrated to be silent (i.e. insertions here are tolerated without fitness cost) in multiple bacterial species¹³⁷. This site represents the high-frequency insertion site for the Tn7 transposon¹³⁸, however, instead of Tn7 transposon mutagenesis, the *lux* operon insertion was achieved by a scarless, markerless homologous recombination approach^{64,66}. The expression of *luxCDABE* in this strain was driven by a strong phage (PA₁) promoter. Given the lack of available data on relative promoter activity in KP I also generated an isogenic strain in which expression of *luxCDABE* was driven by a synthetic Biofab promoter⁶⁷. The Biofab promoter is constitutive in *E. coli*¹³⁹ but the relative promoter strength, compared to the PA₁ phage promoter, was unknown. I started by excluding overt fitness defects in strains tagged with the PA₁ and Biofab driven *luxCDABE* expression (KP PA₁-luxCDABE and KP Biofab-luxCDABE, respectively) and found no overt difference in growth in rich (LB) or minimal (M9) media compared to KP_{WT} (**Figure 3.5B&C**). I assessed the luminescence from KP PA₁-luxCDABE and KP Biofab-luxCDABE over time (**Figure 3.5D**), which demonstrated that the PA₁ promoter had significantly more activity (in both LB and M9) than the Biofab promoter. I therefore chose to proceed with the KP PA₁-luxCDABE strain to evaluate IT delivery and administered KP PA₁-luxCDABE, grown in LB to a stationary phase overnight culture (total dose ~2x10⁸ CFU/50ul inoculation), as this resulted in the highest luminescence output in our assay. 2-dimensional (2D) imaging data were captured and analysed on an IVIS SpectrumCT *in vivo* imaging platform immediately after inoculum administration. This demonstrated that inoculum reached the lungs in all animals with some weak signal visible in the trachea of mice (**Figure 3.5E**). As I had demonstrated that KP PA₁-luxCDABE could be visualised by 2D imaging I proceeded to carry out 3-dimensional (3D) diffuse



light imaging combined with μ CT imaging reconstruction on a single animal (**Figure 3.5F**). This technique requires a longer imaging time (20 min vs 5 minutes) and as I was delivering a high inoculum (total $\sim 2 \times 10^8$ CFU) I only used one mouse for this experiment. The 3D reconstruction demonstrated a distribution deep into the lung parenchyma with the signal confined, as expected, within the thoracic cavity. Transaxial cuts and reconstructions demonstrated bilateral lobar distribution with bioluminescence signal visible deep in the right hemithorax to the level just superior to the right hemidiaphragm (**Figure 3.5F, inset f**).

3.2.11. The 3' *glmS* site is a silent genomic insertion site in *Klebsiella pneumoniae*

Having validated our delivery method, I proceeded to infect mice with 500 CFU of KP_{WT} and enumerate CFU counts from lungs and blood at 36 h post infection (hpi). The dose and time point were chosen as these had been identified in our literature analysis. I started by infecting 10 mice, 5 with KP_{WT} and 5 with $KP_{PA_1-luxCDABE}$ to evaluate if this strain could be used as an *in vivo* imaging tool. KP_{WT} resulted in high bacterial burdens in the lungs of all animals and bacteraemia in 4/5 animals infected (**Figure 3.6A**). $KP_{PA_1-luxCDABE}$ was attenuated in comparison with KP_{WT} , with significantly lower pulmonary burdens with undetectable bacteraemia in all animals. This initial screen suggested that a 500 CFU dose and 36 hpi time point was suitable for female BALB/c infections with KP_{WT} . However, I was unable to ascertain if $KP_{PA_1-luxCDABE}$ was attenuated because the *glmS* insertion site was disrupted or if the $PA_1-luxCDABE$ cargo, with high *luxCDABE* expression, was attenuating the strain. In order to investigate the latter I proceeded to evaluate $KP_{Biofab-luxCDABE}$, as I had shown that the Biofab promoter activity resulted in lower bioluminescent signal *in vitro* (**Figure 3.5D**). I conducted this experiment over two biological repeats, with a total of 10 mice infected with both strains. KP_{WT} resulted in robust infection in all animals with high numbers of bacteria recovered from the lungs and bacteraemia in 6/10 animals. However, $KP_{Biofab-luxCDABE}$ was significantly attenuated with significantly reduced bacterial burdens in the lung and no bacteraemia detected in any mice (**Figure 3.6B**). Taken together these data suggested that *luxCDABE* expression at the *glmS* site, irrespective of expression level attenuates KP *in vivo*.

luxCDABE is a large operon measuring 5.798kb and whilst our data demonstrated that it attenuated KP *in vivo* I was still unable to exclude if this was due to excessive ATP consumption (i.e. the bioenergetic cost of *luxCDABE* expression), *glmS* site disruption or the size of the insertion. I generated two further tagged strains to test this hypothesis and inserted *sfGFP* (KP_{GFP}) and *mRFP1* (KP_{RFP}) genes into the *glmS* site of KP_{WT} . These genes are both smaller than *luxCDABE* (5.798kb) with the *sfGFP* and *mRFP1* ORFs measuring 1.402kb and 0.675kb, respectively. In both cases the expression of the fluorescent protein was driven by the synthetic Biofab promoter (as used in $KP_{Biofab-luxCDABE}$). I tested these *in vitro* and observed no defects in growth and confirmed the expression of fluorescent protein by microscopy (**Figure 3.6C-E**). I then infected 10 mice per group, over 2 biological repeats with 500 CFU KP_{WT} , KP_{GFP} and KP_{RFP} ,

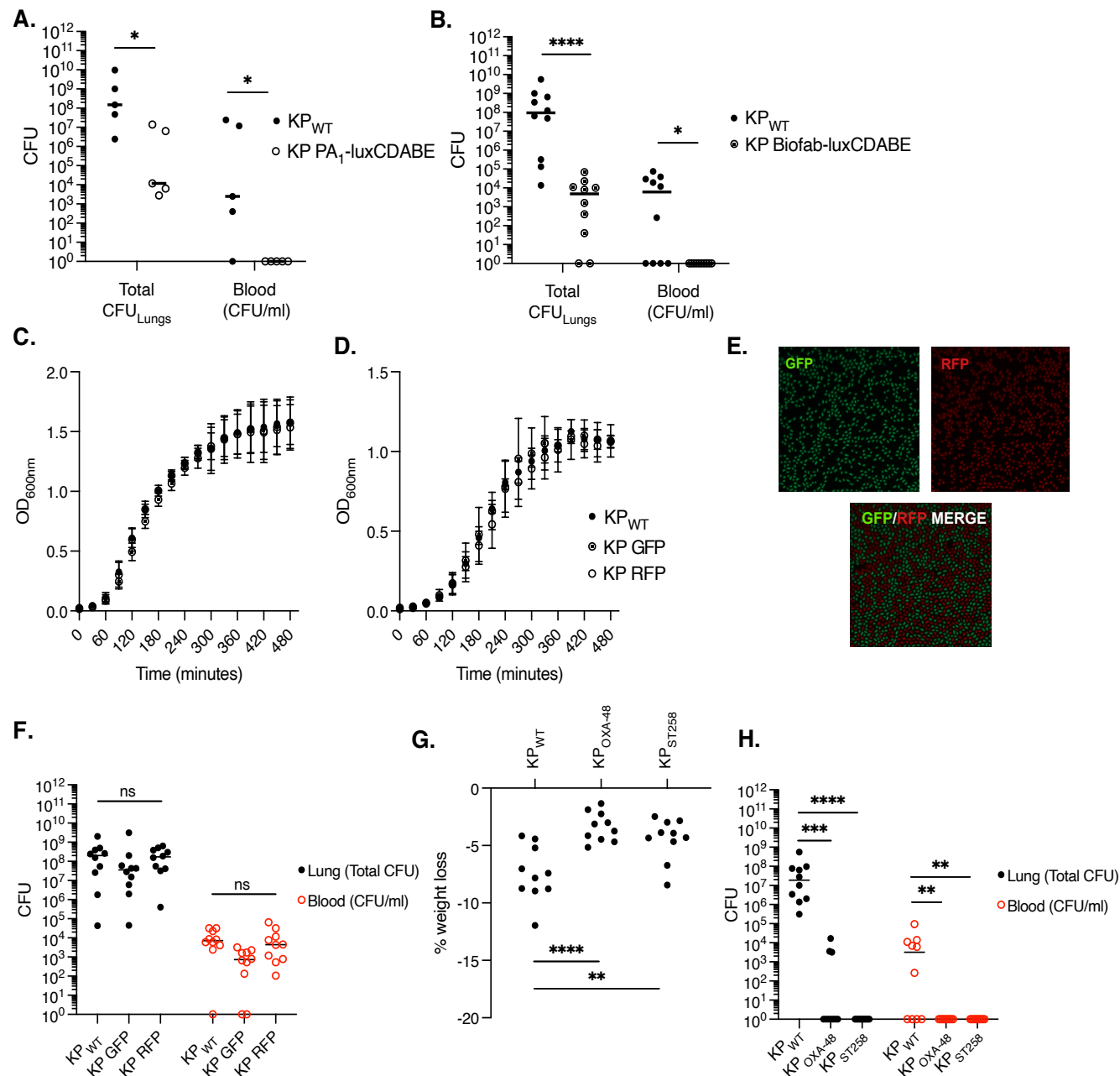
which resulted in robust infection with high lung bacterial burdens and levels of bacteraemia (**Figure 3.6F**). No significant differences were found between the wild-type (KP_{WT}) or tagged strains (KP_{GFP} and KP_{RFP}) indicating that the 3' *glmS* site disruption was tolerated in KP_{WT} . Whilst these experiments were unable to ascertain if it was the size of the *luxCDABE* insertion and or bioenergetic cost of expression that attenuated KP , these experiments provided us with an optimised model with reliable kinetics and two genomic fluorescent protein tags for future work.

3.2.12. Clinical isolates fail to induce robust infections in BALB/c mice

I had previously noted that a wide variety of KP strains (216, **Figure 3.2A**) had been used for *in vivo* infections in mice. I proceeded with an ATCC43816 derivative, due to the large literature base suggesting reliable infection kinetics. I wanted to understand if, having optimised our inoculation methodology, clinical isolates would result in a severe infection phenotype in mice. I obtained 2 carbapenemase-expressing strains from a reference laboratory as these represent a contemporary healthcare threat. One strain was an ST258 strain, expressing KPC-2 (KP_{ST258}) and the other, a strain expressing OXA-48 (of unknown sequence type, KP_{OXA-48}). I infected mice with 500 CFU of KP_{WT} , KP_{ST258} and KP_{OXA-48} and looked at weight loss and bacterial burdens in the lungs and blood at 36 hpi. Whilst some weight loss was observed in both KP_{ST258} and KP_{OXA-48} infections, significantly more was lost following KP_{WT} infection (**Figure 3.6G**). I observed high numbers of KP_{WT} in the lungs and blood at 36 hpi in keeping with our previous findings (**Figure 3.6H**), however the clinical isolates, KP_{ST258} and KP_{OXA-48} , failed to achieve robust infection. In only 3 (3/10) animals infected with KP_{OXA-48} , were low levels of bacteria recovered from the lungs. In line with the pulmonary burdens and failure to achieve infection at a primary focus, I did not recover any KP_{ST258} and KP_{OXA-48} in the blood of infected animals.

These final animal experiments resulted in us using KP_{WT} for all future work carried out in this thesis (Chapters 4&5) to investigate carbapenem resistance mutations, including their precise molecular mechanisms and their associated impacts on *in vivo* infection. All genetic alterations to KP_{WT} take place on the bacterial chromosome and no plasmid-based expression systems were used. Whilst this approach is technically more challenging and time consuming it allowed me to exclude gene dosage effects from multicopy plasmid expression systems. The two-step recombination approach adopted is scarless and therefore, in cases where genes were substituted with different alleles, these are seamlessly integrated into the KP_{WT} genome⁶³.

Figure 3.6 KP_{WT} induces robust infection in female BALB/C mice.



A. 500CFU of KP_{WT} (n=5 mice) and KP PA₁-luxCDABE (n=5 mice) were administered to mice and bacterial counts enumerated from the lungs and blood at 36 hours post infection (hpi). KP_{WT} induced robust infection in all animals in the lungs and bacteraemia (4/5 animals). PA₁ driven luxCDABE expression attenuates KP with significantly reduced bacteria recovered in the lungs and absent bacteraemia. Bacterial counts in the lungs and blood were compared with an unpaired parametric T-test. *, P<0.05; **, P<0.01; ***P<0.001; ****, P<0.0001.

B. 500CFU of KP_{WT} (n=10 mice) and KP Biofab-luxCDABE (n=10 mice) were administered to mice and bacterial counts enumerated from the lungs and blood at 36 hpi. KP_{WT} induced robust infection. Biofab driven luxCDABE expression attenuates KP Biofab-luxCDABE *in vivo*, with significantly reduced bacteria recovered in the lungs and absent bacteraemia. Bacterial counts in the lungs and blood were compared by with an unpaired parametric T-test. *, P<0.05; **, P<0.01; ***P<0.001; ****, P<0.0001. Data from 2 biological replicates.

C&D. Growth curves in LB (**C**) and M9 media (**D**) of KP_{WT} and KP_{WT} encoding sfGFP (KP GFP) or mRFP1 (KP RFP) inserted into the genome at the 3' *glmS* site. Strains were grown in a plate reader at 37 °C and shaking at 200 rpm. Data from 3 biological replicates.

Figure 3.6 figure legend continued.

E. Microscopy of an admixture of KP GFP and KP RFP confirms expression of functional fluorescent protein. Images were captured in both the GFP and RFP channels and then merged.

F. 500CFU of KP_{WT} (n=10 mice), KP GFP (n=10 mice) and KP RFP (n=10 mice) were administered to mice and bacterial counts enumerated from the lungs and blood at 36 hpi. All strains induced robust infection with no significant differences in the numbers of KP recovered in the lungs or blood from infected animals. Bacterial counts were compared by ordinary one-way ANOVA with Tukey's post-test for multiple comparisons between groups. ns= non-significant, p>0.05.

G. 500CFU of KP_{WT} (10 mice), KP OXA-48 (10 mice) and KP ST258 (10 mice) were administered to animals and weight loss was assessed at 36 hpi. KP_{WT} induced significantly more weight loss than both KP OXA-48 and KP ST258. Weight loss was compared by ordinary one-way ANOVA with Tukey's post-test for multiple comparisons between groups. *, P<0.05; **, P<0.01; ***P<0.001; ****, P<0.0001.

H. Lung and blood CFU counts at 36 hpi following the administration of KP_{WT} (n=10 mice), KP OXA-48 (n=10 mice) and KP ST258 (n=10 mice) KP_{WT} administration results in significantly higher bacterial burdens in the lungs and blood with the clinical isolates failing to induce infection. Lung and blood CFU counts were compared by ordinary one-way ANOVA with Tukey's post-test for multiple comparisons between groups. *, P<0.05; **, P<0.01; ***P<0.001; ****, P<0.0001. F, G&H represent data from 2 biological replicates.

3.3. Discussion

Mouse models of KP infection have helped shape our understanding of both host and KP biology and are a vital cornerstone of research in this field. They provide the complex host pathogen background that cannot be modelled through the sole use of *in vitro* techniques. Basic science, in turn, has shaped the scope of these models by affording scientists the ability to carry out complex genetic alterations in both mouse and KP genomes to address clinically relevant scientific questions.

The mice used in the studies analysed demonstrated significant uniformity in sex, age and breed. I propose that the economic feasibility of experiments and the scope of commercially available tools underpins this lack of heterogeneity. Mouse sex was heavily skewed towards female mice, which in turn can be attributed to intrinsic logistical issues associated with co-housing male mice, which often results in aggression and fighting. The use of male mice would inflate the number of cages required to safely house enough animals for experiments. Housing costs of animals are not insignificant, and this may underpin the predominant use of female animals in studies. To the best of my knowledge there are no clear sex differences in human KP disease or any experimental data comparing outcomes of infections in female and male mice. The influence of the observed sex bias on scientific conclusions therefore remains unknown. Several clinical studies and *post hoc* analyses have investigated sex discrepancies in susceptibility and outcomes in the broader context of sepsis, irrespective of infectious aetiology. These have resulted in mixed results with males appearing more likely to develop sepsis¹⁴⁰ but female sex associated with both improved¹⁴¹ and worse¹⁴¹ mortality outcomes. I did note that mixed sexes were used more frequently in the case of GA animals and attribute this to the limited number of animals that become available from locally housed, non-commercially bred mouse lines.

Mice were also infected at a level of relative immaturity likely due to the purchasing of older mice from

commercial suppliers incurring significant additional costs. I used a simplified (linear) calculation to convert mouse age into relative human age and demonstrate that the peak incidence of human disease lies at the opposite end of the spectrum to that of mice experimentally infected with KP. Mouse age has been explored in other pathogens, for example in the case of *Salmonella typhimurium*, older (22-24 month old) C57BL/6 mice had increased levels of colonisation and early weight loss compared to younger mice (4-6 months old)¹⁴². This was attributed to impaired IFN γ and TNF responses and demonstrates, at least in this host pathogen interaction, that age influences experimental infection outcomes. In the absence of comparative data for KP infections it is difficult to assess the impact of uniformly using younger animals in these KP studies.

The diversity of mouse breeds was limited but did include both inbred and outbred mouse strains. C57BL/6 mice appear to be the mouse line of choice for KP infections, especially in the context of GA animals as there are well-defined techniques for the genetic modification of this line including commercial enterprises that specialise in generating custom genetic modifications (e.g. Jackson Laboratories). This outsources such modifications to those with appropriate expertise and enables researchers to benefit from using these animals without generating them in house, with the downside that it increases the reliance on C57BL/6 mice in experimental research models. In one manuscript (excluded from this analysis as inoculation took place via the IP route) a more diverse group of animals was infected¹⁴³. The mice included routine mouse lines and 73 lines from the Collaborative Cross collection, comprising recombinant inbred lines descended from crossing classical experimental mice. Of note there was a significant range in survival durations following challenge, indicating that mouse breed impacts outcomes and, interestingly, classical laboratory mice lines (C57BL/6, BALB/c, C3H/J and DBA/2) were more susceptible to KP infection. As the genetic background of the host affects KP infection outcome, future studies should expand mouse models beyond classical mice lines.

While not uncommon in research, I noticed a widespread lack of confirmatory findings reported by different groups investigating similar hypotheses in animals. This is likely driven on one hand by publication bias, where the lack of novelty once an initial observation is published results in the inability to publish data again, and on the other by ethical considerations. Experimental animals are not limitless and there is certainly an argument for reducing the overall numbers used, when possible, in scientific research. It is also conceivable that many experiments have been replicated by groups and the data not published. However, in the case of one induced co-morbidity, alcohol excess, there are clear and robust data. This was generated by different research groups using a variety of KP strains and mouse breeds, all of which demonstrated negative infection outcomes following alcohol administration which fits squarely with the

increased susceptibility observed in humans¹⁴⁴. It is interesting to note that neutropaenia was induced to facilitate infection and not to investigate susceptibility to KP infection itself by comparison with non-neutropaenic (unconditioned) controls. KP is a significant problem in humans with haematological disorders where neutropaenia is a common complication of treatment or the underlying condition¹⁴³, but animal models were not used to investigate the susceptibility in this patient population.

KP is a diverse species and multidrug resistant cKP associated with hospital infections is driven by high-risk sequence types¹⁴⁵. Our analysis found that ATCC43816 and B5055 strains were most used in animal infections. Of note, both strains have a hvKP phenotype and indeed, ATCC43186 has been used to explore the *rmpADC* locus which drives two key hypervirulence features- hypermucoviscosity³¹ and increased in capsule production³². These studies were not included in our analysis as they were published after the date of our search, but their results show that mutation of this locus results in attenuation in C57BL/6 mouse infection. Unfortunately, the fact that neither ATCC43816 or B5055 belong to high risk STs or to capsule types that are predominant in classical KP strains may limit inferences derived from studies using them. Nonetheless they do readily infect mice and are genetically tractable, making them good options for laboratory studies of KP pathogenesis. Moreover, ATCC43816 and B5055 have LPS O-antigen types (O1) representative of clinical isolates, although there is less variability here in the KP species than that observed in capsule loci³⁶.

There are multiple features that can be measured as infection outcomes and the focus of most studies was bacterial burdens and cytokine/chemokine responses; CFU counts were used almost universally. BALF may be a more appropriate source for measuring CFU counts if translation into patients is a priority, as this most resembles the sputum samples/endotracheal aspirates taken for microbiological sampling in the hospital environment. While spontaneous sputum samples would have the advantage of being serially monitored, these would be impossible to collect in the context of small rodents. Other samples taken in studies for CFU counts included blood, which is commonly sampled in the clinical environment. Although livers and spleens are not routinely tested in KP infected patients, they do provide additional information regarding dissemination in mouse models.

Cytokines and chemokines were measured frequently in biological materials from infected animals, especially from the lung homogenates and BALF. These immune signals tend to be measured experimentally as biomarkers in human clinical trials¹⁴⁶ rather than in the usual course of clinical practice. Here, the pentraxin and acute phase reactant, C-reactive protein (CRP) is a commonly measured inflammatory marker, where levels increase in both infectious and non-infectious inflammatory processes

in patients¹⁴⁷. However, IL-6 was frequently assessed and this represents the primary cytokine inducing hepatic CRP expression and could, therefore, be anticipated as a suitable surrogate¹⁴⁷. Cytokines that have been successfully used as correlates of infection severity in humans^{148,149} and also assessed in these KP models include IL-6, IL-1 β , IFN- γ , IL-17 and IL-22. Encouragingly, these have also been shown to play a major role in the defence against KP infection^{150,151}. Some have also been employed as therapeutic targets in modulating the host response to infection such as the anti-IL-6 therapy, Tocilizumab, in severe COVID-19 disease¹⁵². I note that the measurement of adaptive T-cell and antibody responses, as correlates of protection towards future KP infections were absent, but have both now come to the forefront in light of the current COVID-19 pandemic¹⁵³. However, most studies here were acute infection models and adaptive responses were therefore disregarded unless the experimental intervention was immunisation (e.g. ¹⁵⁴). Whilst one could envisage using the data obtained in these studies to select cytokines suitable as prognostic and/or diagnostic biomarkers, the use of different assay platforms, sample preparation and sampled material makes it difficult to directly compare measured levels between manuscripts.

I found that physiological outcomes were rarely assessed and reported. Whilst some do require additional expensive equipment and expertise (e.g. arterial blood pressure), more simple measures such as weight loss were not routinely reported yet form part of basic laboratory animal practice. Such data may be seen as rudimentary in the field but do provide useful information on the overall disease process and perturbations in physiology induced by infection. Given the subjectivity associated with severity scoring systems it is difficult to directly compare scores reported by different research groups, even using the same system. Despite these limitations our personal view is that measuring physiological parameters is important to determine the impact on the host, especially when it allows monitoring of the state of the animal throughout an infection time course, and with increased use could become routine in research practice. They maximise the information that could be gained from each experimental animal as well as aiding us in the search for a more universal end point for these infections; the lack of which currently complicates data comparisons and interpretation. Whilst the definitions of sepsis and septic shock have changed with time⁶, an assessment of physiology, in the form of respiratory rate, heart rate and/or systolic blood pressure has been incorporated into all accepted consensus definitions^{4,155}. Our KP infection models should aim to measure these if they are to accurately recapitulate the assessment of sepsis in human patients.

In this results chapter I optimise a delivery method to induce severe KP pneumonia in female BALB/c mice, generating a new in-house pre-clinical infection model. Despite highlighting significant homogeneity in the

mice employed, in our literature collection, I chose to proceed with single sex (female) infections. This was due to the economic implications of mixed sex experiments and the inability to cohort animals into single cages, which incurs significant animal husbandry and housing costs. In addition, whilst C57BL/6 mice had been widely used, preceding work in our laboratory had passaged ATCC43816 through female BALB/c mice (to generate our laboratory strain ICC8001/KP_{WT}) and I did not envisage the use of GA animals as part of this project. I anticipated further benefit in the choice of this breed as BALB/c mice are widely used in the vaccinology field¹⁵⁶, which would enable us to exploit this model in the evaluation of urgently needed novel prophylactic KP therapies. I chose 8-10 week old mice, weighing 18-20g, for our infections as these are readily available from commercial suppliers. I noted that, at this age range, the weight of supplied animals was very uniform, which may have also driven the high use of mice around this age in our literature review. Finally, BALB/c mice represented the second most common mouse breed in the literature and this allowed us to draw comparisons in outcome variables, with the published literature, as I built increasing complexity into our model.

Our KP ATCC43816 derivative (ICC8001/KP_{WT}) has been widely adopted as a prototype strain in the field and our future work required a KP strain that was genetically tractable. There were several other logistical advantages to using this strain, including the sensitivity to most antibiotics, which would be required in the selection of genetic mutants. To that end, I show that I could seamlessly introduce the *lux* operon and fluorescent protein encoding genes into the genome of KP_{WT}, and that this process did not attenuate strains *in vitro*. Our growth curve data suggested no KP growth defect with either the PA₁ or Biofab promoter driven *lux* operon expression, however, *in vivo*, I observed significant attenuation. This suggests that the absence of *in vitro* fitness costs cannot exclude fitness cost in the complex host-pathogen interaction provided by murine models. Whilst this precluded the use of either of these strains for tracking KP infection in real-time by *in vivo* imaging, they provided a useful reagent to enable us to validate the intubation-mediated intratracheal delivery system. The delivery system is effective in penetrating deep into the lungs and without significant upper airway soiling. This allowed us to ensure that our model was a true pneumonia model (i.e. intra alveolar infection) rather than an upper airway bronchitis or tracheitis model. The KP GFP and KP RFP infections verified that the 3' *glmS* site can be used for the introduction of exogenous genetic material into the KP_{WT} genome and this did not influence the virulence of these tagged strains *in vivo*.

I also required a KP strain that would induce robust pulmonary infections in mice with dissemination and bacteremia, associated with a septic phenotype, to replicate infections in critically unwell patients¹⁵⁷. Whilst most hospital-acquired KP infections are caused by classical KP strains¹⁴⁵, the mouse infections with

KP_{ST258} and KP_{OXA-48}, both classical KP isolates, indicated that clinical isolates could not be used directly for murine infections in our model. Whilst it would have been beneficial to have additional KP strains to further validate future findings in diverse KP backgrounds, these experiments explain the reliance on ATCC43816 and B5055 in mouse models. Both strains have a hypervirulent phenotype, which is mediated by increased mucoviscosity, increased capsule production and the expression of more siderophores, which may partially underpin their ability to infect the murine host^{31,32,38,158}. Indeed, when mutants in the *rmpADC* locus, which drives the hypermucoviscous and hyperencapsulated phenotype, are administered to mice, it results in attenuation of ATCC43816^{31,32}. Similarly, siderophore mutants demonstrate a failure in dissemination from the lungs into blood, spleens and livers of infected animals³⁸. Of note KP_{ST258} resulted in no recoverable bacteria in the lungs or blood at 36 hpi, however, work was carried out another group identified an ST258 isolate (KP35,¹⁵⁹) that is able to infect mice, albeit at much high inoculum doses (1-2x10⁸ CFU by IN infection) than I was using for our ATCC43816-derived KP_{WT} strain (500 CFU by IT infection).

In summary, I describe the contemporary KP literature, in which experimental infections are induced in the lungs of mice. I critically appraise experimental practice and use this as a basis to develop an in-house preclinical model to investigate KP biology *in vivo*. This model is based on female, 18-20g, 8-10 week old BALB/c mice, infected with the ATCC43816-derived ICC8001/KP_{WT} strain, which I was able to genetically manipulate. To that end, I tested two tagged strains KP GFP and KP RFP, which will be used as key reagents to test experimental hypotheses in the following chapter.

Results Chapter 4. An OmpK36 L3 Glycine-Aspartate insertion mediates pore constriction.

4.1. Introduction

Carbapenem resistant KP (CRKP) has been consistently listed as a critical priority organism for which new drugs are urgently needed¹⁶⁰. The primary mechanism by which this resistance phenotype is achieved is the acquisition of plasmid encoded carbapenemase enzymes, which inactivate carbapenems in the periplasm by hydrolysis^{161,162}. Ambler Class A (KPC-variants), B (NDM-variants) and D (OXA-48-like) enzymes are particularly prevalent in the KP species and demonstrate strong associations with high-risk multidrug resistant sequence types (ST)¹⁶³. The most notable example of this relationship is KPC-variant enzymes in ST258 KP strains, which have spread globally, and are now endemic in many countries including Israel¹⁶⁴ and Italy¹⁶⁵. Whilst other strong associations exist, such as ST16 with NDM-1 enzymes, and ST231 and OXA-232, these are underreported in the literature, as these STs predominate in lower and middle-income countries, which have less capacity for sequencing and surveillance^{166,167}. Resistance plasmid acquisition, by horizontal gene transfer, is followed by clonal expansion and vertical transmission of carbapenemase-encoding plasmids within STs¹⁶⁶. In the case of OXA-48-like enzymes in KP, a particularly promiscuous plasmid background, pOXA48a-like, has a depressed conjugation system leading to rapid spread amongst multiple STs in locoregional CRKP outbreaks¹⁶⁸.

In addition to plasmid-borne carbapenemase enzymes, chromosomal mutations have evolved under strong antibiotic selection, to further increase the levels of carbapenem resistance achieved in KP and other nosocomial multidrug resistant threats¹⁶⁹. These mutations occur in non-selective outer membrane porins, which allow the diffusion of hydrophilic substrates, across the otherwise impermeable lipophilic membrane^{66,170}. In homeostasis, these allow the influx of nutrients and efflux of waste products into and out of the periplasmic space, respectively¹⁷¹. However, they also represent the primary mechanism by which important classes of antibiotics, such as beta-lactams, cephalosporins and carbapenems enter and reach their site of action, transpeptidase enzymes, in the periplasm. The prototypical non-selective porins, OmpF and OmpC, in *Escherichia coli* have been most studied¹⁷². They are differentially expressed and OmpF is found at lower abundance than OmpC, with $>10^5$ porin monomers estimated to be present in the outer membrane of each *E. coli* cell¹⁷³. Orthologues of OmpF and OmpC are present in related Enterobacterales, including OmpEc35 and OmpEc36 in *Enterobacter cloacae* and OmpK35 and OmpK36 in KP¹⁶⁹. All these porins form hydrophilic water filled pores, composed of 16 β -strand barrels, that traverse the outer membrane with intracellular and extracellular loops connecting adjacent strands¹⁶⁹. A conserved feature of all members of this family is an extended extracellular loop 3 (L3), which, in its natural

conformation, folds back, forming a constriction point, at the midpoint of the barrel⁶⁰. This regulates the luminal size, determining both size exclusion and rate of diffusive capacity. One mechanism by which reduced antibiotic diffusion can be achieved is by the genetic truncation of these proteins which has been reported for OmpF, OmpC, OmpE35, OmpE36, OmpK35 and OmpK36¹⁶⁹. However, in KP, it was recognised that amino acid insertions in L3 of OmpK36 were associated with increased carbapenem resistance in sporadic case reports of clinical isolates. The first such report was in 2010 and described the presence of an OmpK36 variant containing a threonine-aspartate (TD) L3 insertion. This conferred a carbapenem (ertapenem) resistant phenotype to a non-specialised extended-spectrum-beta-lactamase (CTX-M-15) expressing strain¹⁷⁴. This was followed by a report in 2012 of a glycine-aspartate (GD) L3 insertion in an OmpK36 variant encoded by an ST258 strain in Italy¹⁷⁵. The ST258 strain had a high meropenem minimum inhibitory concentration (MIC) and expressed the carbapenemase KPC-3. Finally, in 2013, a group looked at ST258 KP infections in solid organ transplant patients and noted that L3 GD insertions were present in 8 of 23 sequenced strains¹⁷⁶. This indicated that the L3 OmpK36 L3 GD insertions may be more common than the sporadic case reports in the literature suggested.

At the inception of this project, no large KP genome collections were available to prospectively investigate the role of OmpK35 and OmpK36 on carbapenem resistance utilising a genomics approach. However, such collections have now become available and, indeed, some incorporate both genomic and phenotypic metadata (antibiotic MICs) for each isolate which has made this approach feasible (EuSCAPE¹⁴⁵). I wanted to investigate ST258 OmpK35 and OmpK36 porins in detail as this clonal lineage was highly successful and the literature reports suggested that modification in the genes encoding this porin variants may be prevalent¹⁷⁶. In the United Kingdom the first KPC-positive ST258 KP strain was identified in 2007 in Scotland¹⁷⁷ and until 2010 isolates were only reported on an occasional basis. From 2010 a multi-ST outbreak of KPC-producing KP occurred in the North West of England in which ST258 isolates predominated (19/33 sequenced isolates)^{178,179}. I obtained a clinical ST258 isolate (KP_{ST258}) from this outbreak, with a high meropenem MIC, and used this as the basis to investigate ST258 modifications in both OmpK35 and OmpK36 on carbapenem resistance and virulence *in vivo*.

4.1.1. Specific chapter aims:

1. To define the precise molecular mechanism by which ST258 OmpK35 and OmpK36 variants increase carbapenem resistance.
2. To evaluate the role that ST258 OmpK35 and OmpK36 modifications have on the virulence of KP *in vivo*.

4.2. Results

4.2.1 ST258 OmpK35 and OmpK36 porin configurations increase carbapenem resistance

I obtained a KPC-2 positive ST258 KP strain (KP_{ST258}) from the antimicrobial resistance reference laboratory at Public Health England. The strain was collected as part of an infection control and surveillance effort to contain a large outbreak of CRKP in the North West of England¹⁷⁸. The KPC-2 encoding plasmid was extracted from KP_{ST258} and transformed into a laboratory *E. coli* strain. Purified plasmid was sequenced, by long-read PacBio sequencing, and the plasmid found to have the identical sequence to a 113.6kb KPC-2 encoding pKpQIL-UK plasmid (Genbank accession: KY798507). No other functional antimicrobial resistance determinants were present on pKpQIL-UK.

I wanted to understand the role that the two major KP outer membrane porins, OmpK35 and OmpK36, played in carbapenem resistance. To do this, I cloned the open reading frame (ORF) of *ompK35* and *ompK36* from the clinical KP_{ST258} isolate and substituted them in place of the respective wild-type (WT) ORFs in our laboratory strain KP_{WT}. All strains and gene variants used in this chapter, including attributes, can be found in **Tables 4.1A&B and 4.2**.

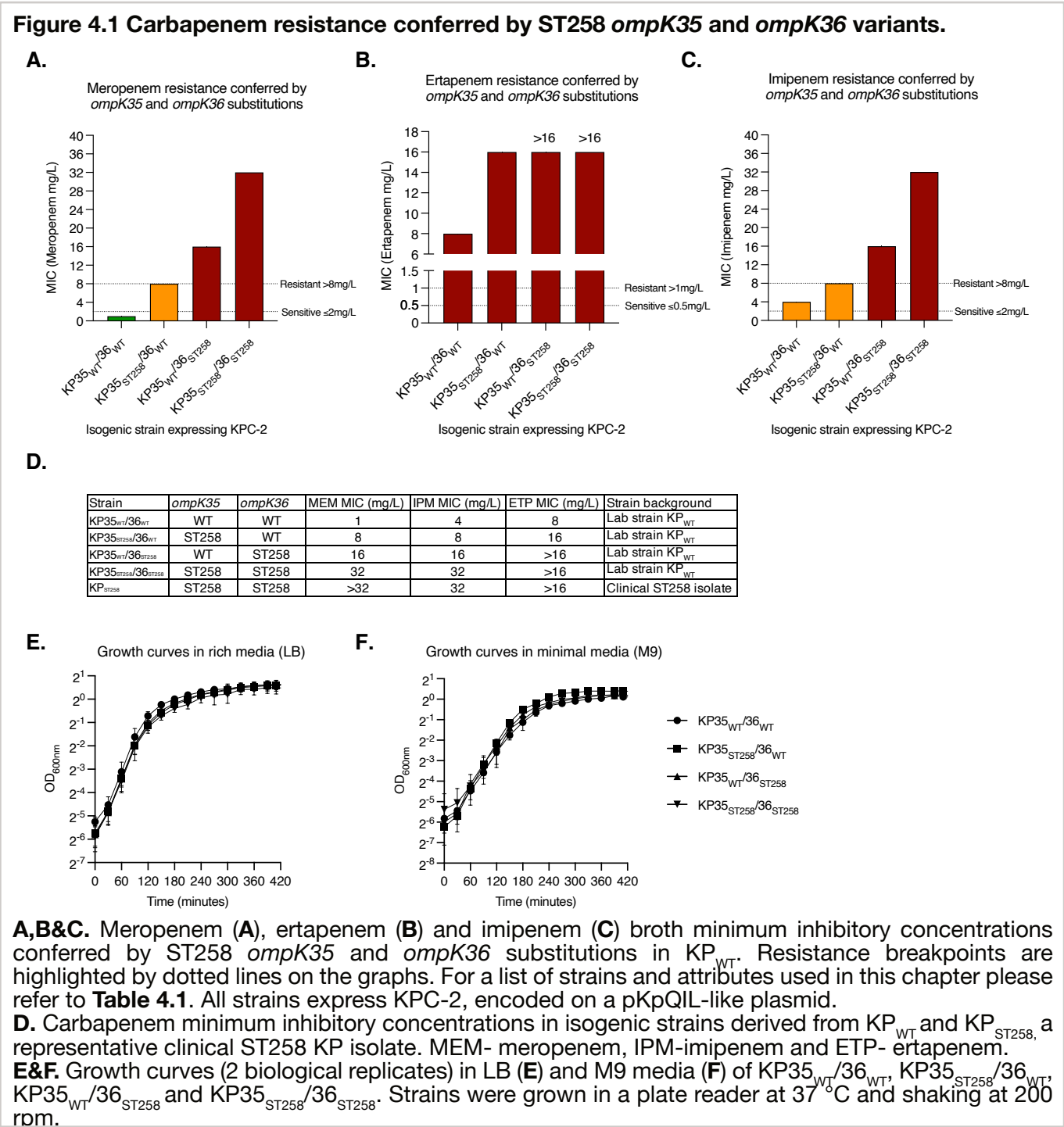
Table 4.1A <i>ompK35</i> variants used in this chapter.			
<i>ompK35</i> genes	OmpK35 protein variant	L3 region	Notes
<i>ompK35</i> _{WT}	OmpK35 _{WT}	WT	Encoded by KP _{WT}
<i>ompK35</i> _{ST258}	OmpK35 _{ST258}	WT	Encoded by KP _{ST258} (truncation and non-functional porin)

Table 4.1B <i>ompK36</i> variants used in this chapter.			
<i>ompK36</i> genes	OmpK36 protein variant	L3 region	Notes
<i>ompK36</i> _{WT}	OmpK36 _{WT}	No L3 insertion (WT)	Encoded by KP _{WT}
<i>ompK36</i> _{ST258}	OmpK36 _{ST258}	Gly-Asp insertion	Encoded by KP _{ST258}
<i>ompK36</i> _{WT+GD}	OmpK36 _{WT+GD}	Gly-Asp insertion	Mutant with Gly-Asp L3 encoded on <i>ompK36</i> _{WT} background
<i>ompK36</i> _{ST258ΔGD}	OmpK36 _{ST258ΔGD}	No L3 insertion	Mutant with Gly-Asp L3 deleted on <i>ompK36</i> _{ST258} background
<i>ompK36</i> _{ST258(R127A)}	OmpK36 _{ST258(R127A)}	Gly-Asp insertion	Arg127 to Ala127 mutation on <i>ompK36</i> _{ST258} background
<i>ompK36</i> _{ST258ΔLSP}	OmpK36 _{ST258ΔLSP}	Gly-Asp insertion	Mutant with Lys165-Ser166-Pro167 L4 deleted on <i>ompK36</i> _{ST258} background

Table 4.2 <i>Klebsiella pneumoniae</i> strains used in this chapter.			
Strain name	<i>ompK35</i>	<i>ompK36</i>	Notes
KP _{ST258}	<i>ompK35</i> _{ST258}	<i>ompK36</i> _{WT}	Clinical ST258 isolate
KP35 _{WT} /36 _{WT}	<i>ompK35</i> _{WT}	<i>ompK36</i> _{WT}	Our parental laboratory strain KP _{WT}
KP35 _{ST258} /36 _{WT}	<i>ompK35</i> _{ST258}	<i>ompK36</i> _{WT}	Otherwise isogenic on KP _{WT} background
KP35 _{WT} /36 _{ST258}	<i>ompK35</i> _{WT}	<i>ompK36</i> _{ST258}	Otherwise isogenic on KP _{WT} background
KP35 _{ST258} /36 _{ST258}	<i>ompK35</i> _{ST258}	<i>ompK36</i> _{ST258}	Otherwise isogenic on KP _{WT} background
KP35 _{ST258} /36 _{WT+GD}	<i>ompK35</i> _{ST258}	<i>ompK36</i> _{WT+GD}	Otherwise isogenic on KP _{WT} background
KP35 _{ST258} /36 _{ST258ΔGD}	<i>ompK35</i> _{ST258}	<i>ompK36</i> _{ST258ΔGD}	Otherwise isogenic on KP _{WT} background
KP35 _{ST258} /36 _{ST258(R127A)}	<i>ompK35</i> _{ST258}	<i>ompK36</i> _{ST258(R127A)}	Otherwise isogenic on KP _{WT} background
KP35 _{ST258} /36 _{ST258ΔLSP}	<i>ompK35</i> _{ST258}	<i>ompK36</i> _{ST258ΔLSP}	Otherwise isogenic on KP _{WT} background
KP35 _{ST258} /Δ36	<i>ompK35</i> _{ST258}	Δ(ORF deletion)	Otherwise isogenic on KP _{WT} background

In order to evaluate the role of each gene I generated three isogenic strains; in the first the *ompK35* gene was replaced (KP35_{ST258}/36_{WT}), in the second the *ompK36* gene was replaced (KP35_{WT}/36_{ST258}) and in the final strain both *ompK35* and *ompK36* genes were replaced (KP35_{ST258}/36_{ST258}). I only replaced the ORF, so that the upstream (5' promoter and ribosome binding site) and downstream (3' transcriptional

terminator) sequences remained identical and intact, ensuring the same regulatory networks governed WT and ST258 *ompK35* and *ompK36* gene expression. I introduced the pKpQIL-UK plasmid from the *E. coli* strain by conjugation into this isogenic collection and assayed resistance to three carbapenems (meropenem (MEM), ertapenem (ERT) and imipenem (IPM)) (**Figure 4.1A-D**) by broth microdilution. In the presence of WT *ompK35* and *ompK36* (KP35_{WT}/36_{WT}, i.e. our KP_{WT} strain), the carbapenem MICs were low, despite KPC-2 expression. In all cases the substitution of either *ompK35*_{ST258} (KP35_{ST258}/36_{WT}) or *ompK36*_{ST258} (KP35_{WT}/36_{ST258}) increased the resistance to MEM, ERT and IPM. However, I noted that the ST258 substitution. For example, when assessing MEM resistance there was a 16-fold increase in MIC



between KP35_{WT}/36_{ST258} and KP35_{WT}/36_{WT} but only 8-fold increase in MEM MIC observed between KP35_{ST258}/36_{WT} and KP35_{WT}/36_{WT} (**Figure 4.1A**). When both loci were substituted (KP35_{ST258}/36_{ST258}) the resulting MICs were the highest at 32mg/L for both MEM and IPM. In the case of ETP, both *ompK36*_{ST258} porin substitutions increased the MIC above the range of the assay (>16mg/L, **Figure 4.1B**). Of note, KP35_{ST258}/36_{ST258} phenocopied the MEM/ERT and IPM MIC profile of KP_{ST258}, demonstrating the key role that these porins play in the generation of a robust carbapenem resistant phenotype in clinically successful isolates (**Figure 4.1D**).

I proceeded to check if the ST258 substitutions resulted in an *in vitro* growth defect and observed no discernible differences in growth in either rich (LB) or minimal (M9 with 0.4% glucose w/v) media with single *ompK35*_{ST258}, *ompK36*_{ST258} or combined *ompK35*_{ST258} and *ompK36*_{ST258} gene replacements (**Figure 4.1E&F**).

4.2.2. Comparative analysis of wild-type and ST258 OmpK36 variants

Having established that both *ompK35*_{ST258} and *ompK36*_{ST258} increased the resistance to carbapenems, I wanted to elucidate the precise underlying molecular mechanism. I started by sequencing both genes from the ST258 strain (KP_{ST258}) and our WT (KP_{WT}) strain and compared the amino acid (aa) sequence by alignment (**Figure 4.2A&B**). In the case of *ompK35* I found that the *ompK35*_{WT} encodes a 337 amino acid (aa) mature protein (following 21 aa Sec-dependent signal sequence cleavage) and the crystal structure of this exact protein had previously been solved⁶⁰. This work showed that OmpK35 adopts a classical non-selective porin architecture, consisting of 16-stranded β-barrels, forming a hydrophilic water filled lumen in the outer-membrane (OM). A constriction point is formed at the midpoint of the barrel by extracellular loop 3 (L3) that determines the minimal pore diameter. In the case of OmpK35_{WT} the minimal pore radius was determined to be 3.6 ± 1.4 Å (pdb:5077⁶⁰). In examining the sequence of *ompK35*_{ST258} I found a frameshift mutation, resulting in a premature stop (TGA) codon which resulted in a highly truncated protein (66aa ST258 vs 337 aa WT, **Figure 4.2A**). The frameshift results in only 19aa of homology to OmpK35_{WT}, precluding the formation of a functional pore. This exact mutation (Genbank accession: FJ577672) is ubiquitous in ST258 strains and present in the earliest sequenced isolates²³.

I then examined the *ompK36*_{ST258} sequence from KP_{ST258} and compared this to our *ompK36*_{WT} sequence from KP_{WT}. The ST258 allele is predicted to encode a full-length mature protein of 348 aa with 94.5% identity to OmpK36_{WT} (344 aa). OmpK36_{ST258} was found to contain two insertions in comparison to OmpK36_{WT}. The first consisted of Gly115-Asp116 (GD) insertion in extracellular L3 and the second a Leu165-Ser166-Pro167 (LSP) insertion in extracellular loop 4 (L4). In addition, the OmpK36_{WT} contained one additional residue (Arg306) that was not present in the OmpK36_{ST258} (**Figure 4.2B**). The OmpK36_{ST258}

A.



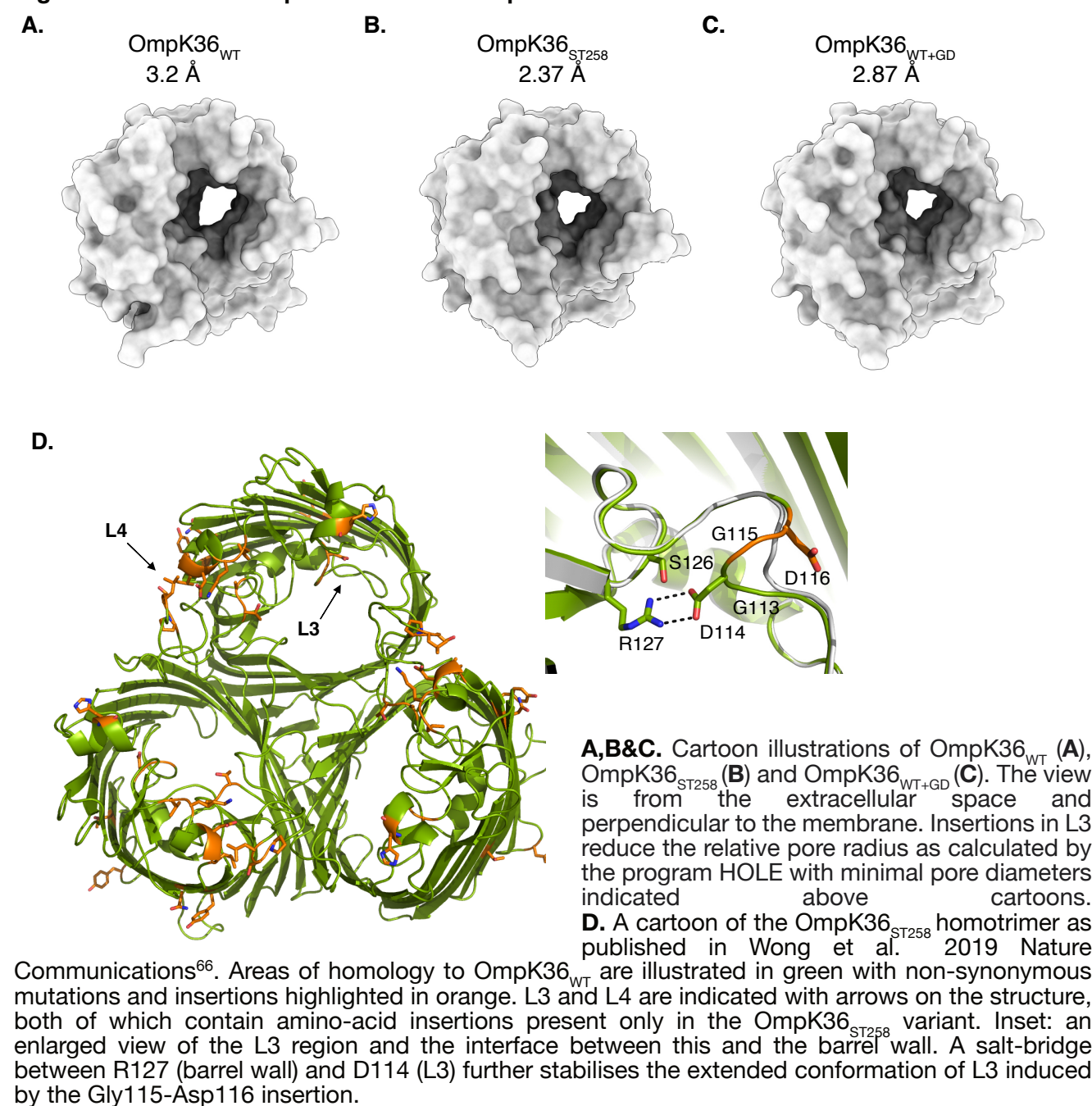
Western blot analysis showing the expression of OmpA and OmpK35/OmpK36 in *E. coli* strains. The strains are KP35-WT/36-WT, KP35-WT/36-WT/36^{WT}, KP35-WT/36^{WT}, and KP35-WT/36^{WT}/36^{WT}. The blot shows bands for OmpA (top) and OmpK35/OmpK36 (bottom). The OmpA band is present in all strains, while the OmpK35/OmpK36 band is only present in the KP35-WT/36^{WT} strain.

. 2019 Nature Communications⁶⁶. o has non-functional porin. *ompK36*_{ST258} encode a functional OmpK36 porin.

I proceeded to confirm that the *ompK35*_{ST258} truncation resulted in absent OmpK35 in KP35_{ST258}/*36*_{WT} and KP35_{ST258}/*36*_{ST258} and that OmpK36 expression was not significantly impacted by the *ompK36*_{WT} to *ompK36*_{ST258} substitution. To that end, I grew cultures of our isogenic porin mutants in minimal media (M9 containing 0.4%w/v glucose) as this has previously been shown to enhance OmpK35 expression¹⁸⁰, isolated OM proteins and separated them by SDS-PAGE. I stained for protein with Coomassie, as there are no commercially available anti-OmpK35 or anti-OmpK36 antibodies for immunoblotting (**Figure 4.2C**). I observed, as anticipated, a band corresponding to OmpA was present in all 4 isogenic strains that appeared unchanged in abundance by the genetic manipulation of either *ompK35* or *ompK36*. OmpK35 was present in KP35_{WT}/*36*_{WT} and KP35_{WT}/*36*_{ST258} and the density was unchanged by the presence of either OmpK36_{WT} or OmpK36_{ST258} expression. In all strains a band corresponding to OmpK36 was present, at a high and unaltered expression level, irrespective of whether OmpK36_{WT} or OmpK36_{ST258} was encoded in the genome. The absence of OmpK35 in KP35_{ST258}/*36*_{WT} and KP35_{ST258}/*36*_{ST258} explained the observed changes in MEM and IPM MIC (**Figure 4.1A-D**) however, the absence of overt changes in OmpK36 abundance suggested that non-synonymous coding mutations or insertions in OmpK36_{ST258} were contributing to the increase in carbapenem resistance.

I wanted to understand the impact that the changes in coding sequence had on the structure of OmpK36_{ST258} (pdb: 6RCP) and OmpK36_{WT} (pdb: 6RD3). I did this by collaborating with the Beis laboratory (Imperial College London) to solve both structures by X-ray crystallography and subsequent related biochemical assays (**Figure 4.3 A-C**). Whilst an *in silico* approach to this may now be possible by using bioinformatic tools, these were not available at the time the work was conducted. Proteins were expressed, purified, crystallised and structures determined by members of the Beis laboratory⁶⁶. Together, we started by comparing our OmpK36_{WT} structure with that of the published OmpK36_{WT} structure (containing the Q235R substitution) and found that this mutation had no effect on L3 conformation. The two structures could be superimposed with a root mean square deviation (rmsd) over 340 carbon atoms (340Cα) of only

Figure 4.3 Structural implications of L3 OmpK36 insertions.



0.18 Å, indicating significant conformational similarity. The overall OmpK36_{ST258} structural conformation was also similar to our OmpK36_{WT} structure (rmsd 0.43 over 340Cα). However, one striking difference was the extended conformation adopted by L3 in OmpK36_{ST258} which was mediated, in part, by the di-amino acid GD insertion. In assessing the minimal pore radius we found a 26% pore constriction (3.2 Å OmpK36_{WT} vs 2.37 Å OmpK36_{ST258}) indicating that the insertion significantly impacted on the size of the OmpK36 pore lumen. In addition, the structural findings suggested that the extended L3 was further stabilised by a supramolecular interaction between L3 and the opposing barrel face, by the formation of a salt bridge between Asp114 (in L3) and Arg127(R127) (in the barrel). The L4 LSP insertion was clearly

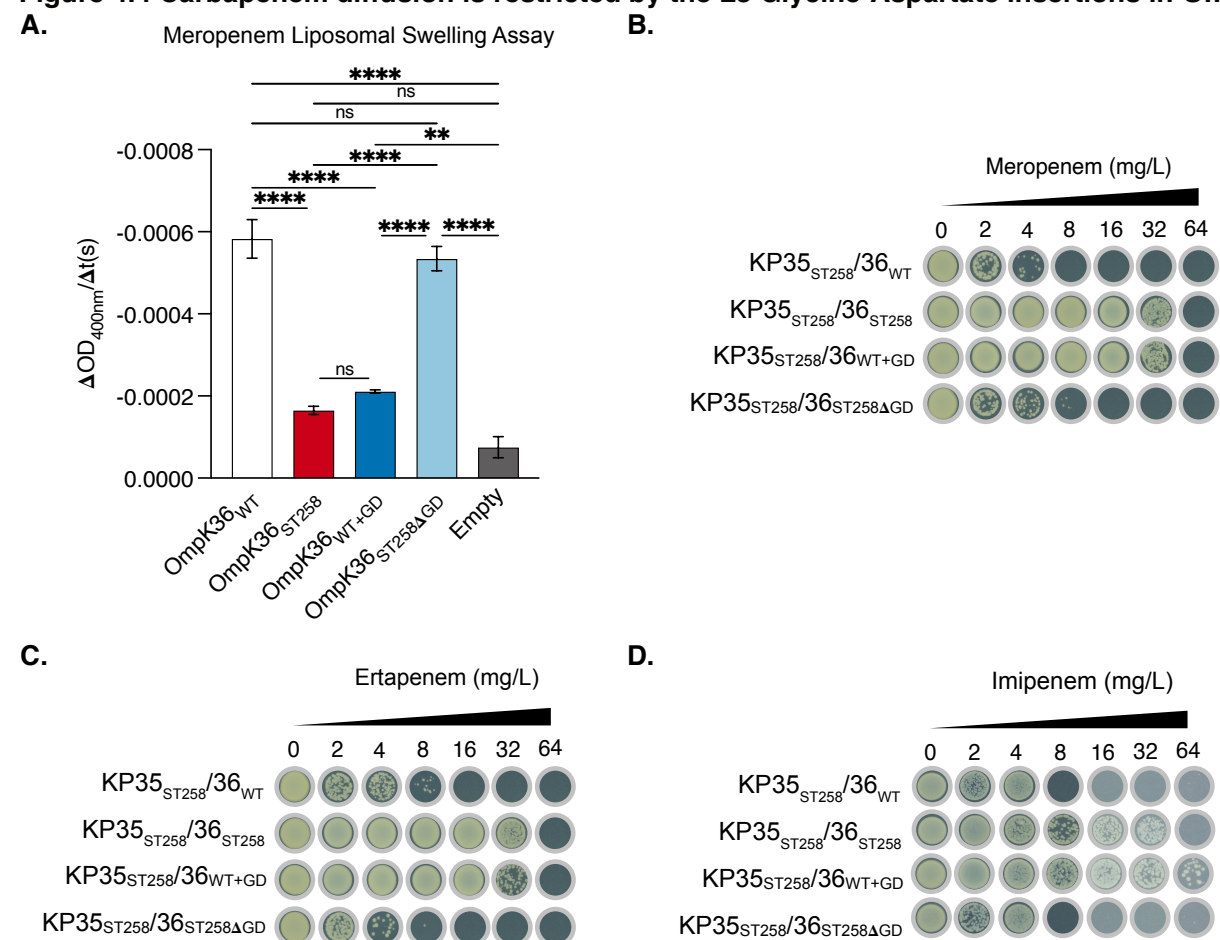
visible as an extended loop but this did not impact on the monomer interactions and OmpK36_{ST258} like OmpK36_{WT} naturally formed homotrimeric complexes. We proceeded to generate a chimeric mutant, in which we inserted a GD into the L3 of OmpK36_{WT} that we called OmpK36_{WT+GD}. We list the OmpK36 variants investigated in this work in **Table 4.1**. This allowed us to investigate the role of the L3 GD insertion, independently of the other changes observed between OmpK36_{WT} and OmpK36_{ST258} (**Figure 4.2B**) to allow for more direct comparisons. The pore radius in OmpK36_{WT+GD} was also significantly reduced at 2.87 Å, representing a 10.3% reduction in pore diameter compared to OmpK36_{WT} (**Figure 4.2C**). We also noted the formation of a D114/R127 salt-bridge in OmpK36_{WT+GD} stabilising the extended L3 conformation. These findings indicated that the GD L3 insertion mediated a reduction in pore diameter in both the OmpK36_{ST258} and OmpK36_{WT+GD} isoforms but did not exclude other subtle effects of sequence variation we had observed between OmpK36_{ST258} and OmpK36_{WT}. We proceeded to assess the role of the L3 GD insertion, L4 LSP insertion and D114/R127 salt-bridge in a series of biochemical functional assays.

4.2.4. Carbapenem diffusion is restricted by the L3 Glycine-Aspartate insertion in OmpK36_{ST258}

We started by investigating the role of the L3 GD insertion and, in addition to the chimeric OmpK36_{WT+GD}, we generated an OmpK36_{ST258} mutant in which we deleted the L3 GD insertion (OmpK36_{ST258ΔGD}). We tested our series of OmpK36 L3 mutants in two assays; meropenem diffusion across purified OmpK36 variants by liposome swelling assay and functionally by assessing the impact of their expression in isogenic KP strains on meropenem MIC. In the MIC assays, we replaced the *ompK36* ORF with our mutant *ompK36* gene of interest, on an *ompK35*_{ST258} truncation background, and in tandem with KPC-2 expression from the pKpQIL-UK plasmid.

The meropenem liposomal swelling assays were conducted by reconstituting liposomes with purified recombinant OmpK36 variants. Empty liposomes were used as a control to account for meropenem diffusion in the absence of any protein. Liposomal swelling occurs as meropenem diffuses into the spherical multilamellar liposomes and we detected the increase in volume by a reduction in optical density (OD_{400nm}). OD_{400nm} readings were taken at 1 second (s) intervals and we calculated the rate of swelling (ΔOD_{400nm}/Δt(s)) to compare the diffusion capacity of each OmpK36 variant (**Figure 4.4A**). Diffusion of meropenem occurred at the highest rate in OmpK36_{WT}-containing liposomes. We observed that the deletion of the L3 GD, in OmpK36_{ST258ΔGD}, also resulted in the same rate, with no significant difference between these two proteins. Conversely, OmpK36_{ST258} resulted in a low level of diffusion and the insertion of the L3 GD in OmpK36_{WT+GD} replicated this reduction in diffusion compared to OmpK36_{WT}. These results suggest that L3 GD mediates pore constriction, which in turn determines meropenem diffusion rate across the OmpK36 porin. I then conducted meropenem agar dilution assays to assess the resistance achieved by the expression of these OmpK36 variants. KP35_{ST258}/36_{WT} had KP35_{ST258}/36_{ST258} had low and high levels

Figure 4.4 Carbapenem diffusion is restricted by the L3 Glycine-Aspartate insertions in OmpK36.

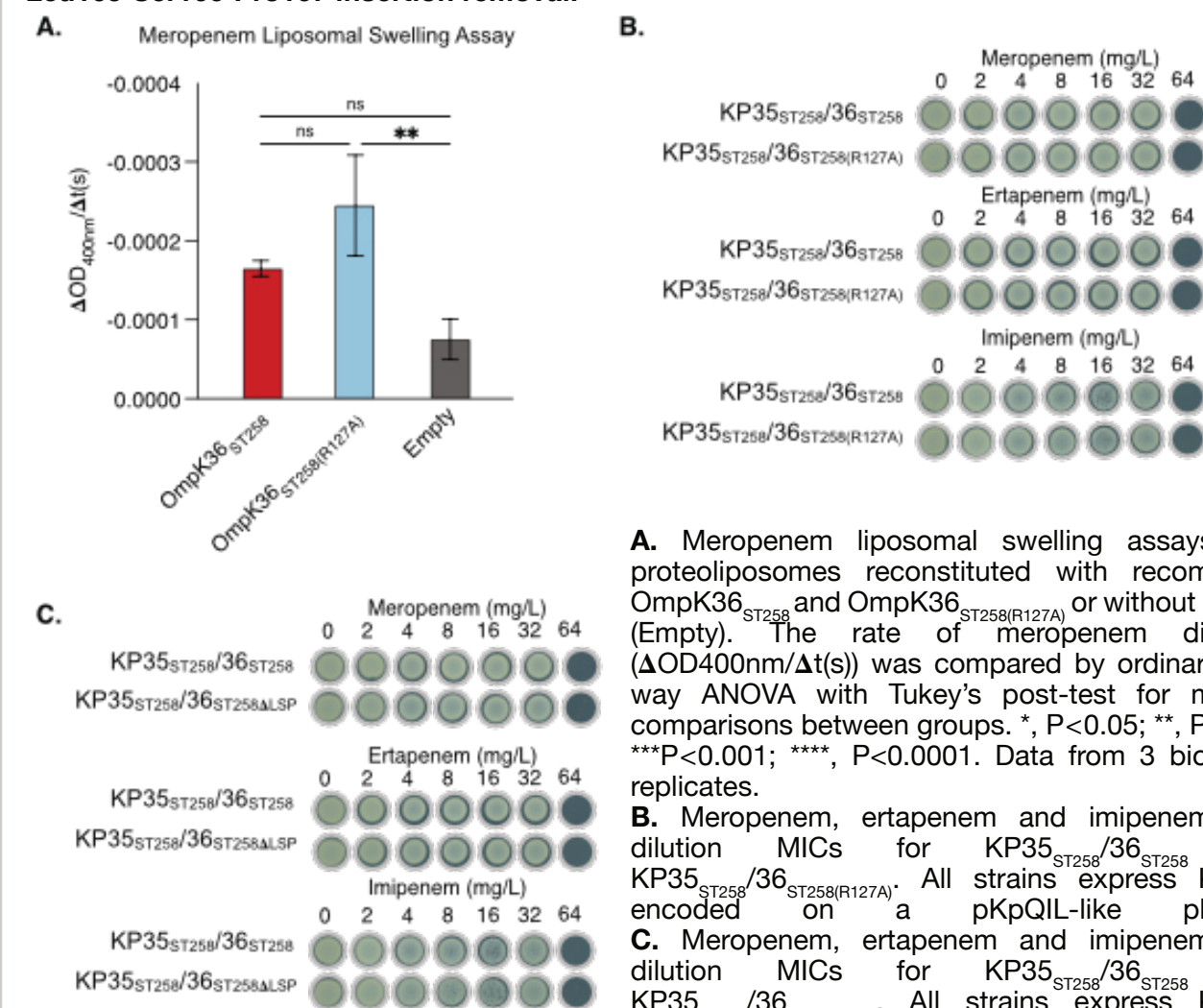


A. Meropenem liposomal swelling assays with proteoliposomes reconstituted with recombinant OmpK36_{WT}, OmpK36_{ST258}, OmpK36_{WT+GD}, OmpK36_{ST258ΔGD} or without protein (Empty). The rate of meropenem diffusion ($\Delta OD_{400nm}/\Delta t(s)$) was compared by ordinary one-way ANOVA with Tukey's post-test for multiple comparisons between groups. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Data from 3 biological replicates).

B,C&D. Meropenem (**A**), ertapenem (**B**) and imipenem (**C**) agar dilution MICs for KP35_{ST258}/36_{WT}, KP35_{ST258}/36_{ST258}, KP35_{ST258}/36_{WT+GD} and KP35_{ST258}/36_{ST258ΔGD}. All strains express KPC-2, encoded on a pKpQIL-like plasmid. Representative images from 2 biological replicates.

of meropenem resistance in keeping with our previous broth MIC data (**Figure 4.4B**). The insertion of the L3 GD in KP35_{ST258}/36_{WT+GD} phenocopied the resistance of KP35_{ST258}/36_{ST258} and the deletion of L3 GD in KP35_{ST258}/36_{ST258ΔGD} reverted the MIC to that observed in KP35_{ST258}/36_{WT}. Taken together these results suggest that biochemically and functionally the L3 GD insertion mediates the increase in meropenem resistance conferred by OmpK36_{ST258}. I confirmed that this effect extended to ERT and IPM and found high resistance levels in KP35_{ST258}/36_{ST258} and KP35_{ST258}/36_{WT+GD} with low resistance levels in KP35_{ST258}/36_{WT} and KP35_{ST258}/36_{ST258ΔGD} (**Figure 4.4C&D**).

Figure 4.5 Carbapenem diffusion is not affected by R127/D114 salt-bridge disruption or L4 Leu165-Ser166-Pro167 insertion removal.



A. Meropenem liposomal swelling assays with proteoliposomes reconstituted with recombinant OmpK36_{ST258} and OmpK36_{ST258(R127A)} or without protein (Empty). The rate of meropenem diffusion ($\Delta OD_{400nm}/\Delta t(s)$) was compared by ordinary one-way ANOVA with Tukey's post-test for multiple comparisons between groups. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Data from 3 biological replicates.

B. Meropenem, ertapenem and imipenem agar dilution MICs for KP35_{ST258}/36_{ST258} and KP35_{ST258}/36_{ST258(R127A)}. All strains express KPC-2, encoded on a pKpQIL-like plasmid. **C.** Meropenem, ertapenem and imipenem agar dilution MICs for KP35_{ST258}/36_{ST258} and KP35_{ST258}/36_{ST258ΔLSP}. All strains express KPC-2, encoded on a pKpQIL-like plasmid. Representative images from 2 biological replicates.

4.2.5. The OmpK36_{ST258} Aspartate114/Arginine127 salt-bridge and L4 Lysine165-Serine166-Proline167 insertion do not impact on meropenem resistance.

The structural findings suggested the formation of a D114/R127 salt-bridge that stabilised the extended L3 conformation in both OmpK36_{ST258} and OmpK36_{WT+GD} (**Figure 4.3D**). Whilst our findings had implicated the L3 GD insertion as the major feature driving pore constriction, we wanted to understand the role that this salt-bridge played on resistance. To that end we generated a mutant in OmpK36_{ST258} in which R127 was mutated to an alanine OmpK36_{ST258(R127A)}. This substitution precluded salt-bridge formation, as this relies on the presence of a cationic guanidinium ($RNHC(NH_2)_2^+$ arginine (R127) side chain. We mutated R127, instead of D114, as this maintained the L3 GD insertion. When we biochemically assessed meropenem diffusion by liposomal swelling assay, we found no significant differences between OmpK36_{ST258} and OmpK36_{ST258(R127A)} indicating that the salt-bridge did not reduce meropenem diffusion (**Figure 4.5A**). In keeping with this, meropenem resistance was the same in the isogenic strain pair KP35_{ST258}/36_{ST258} and

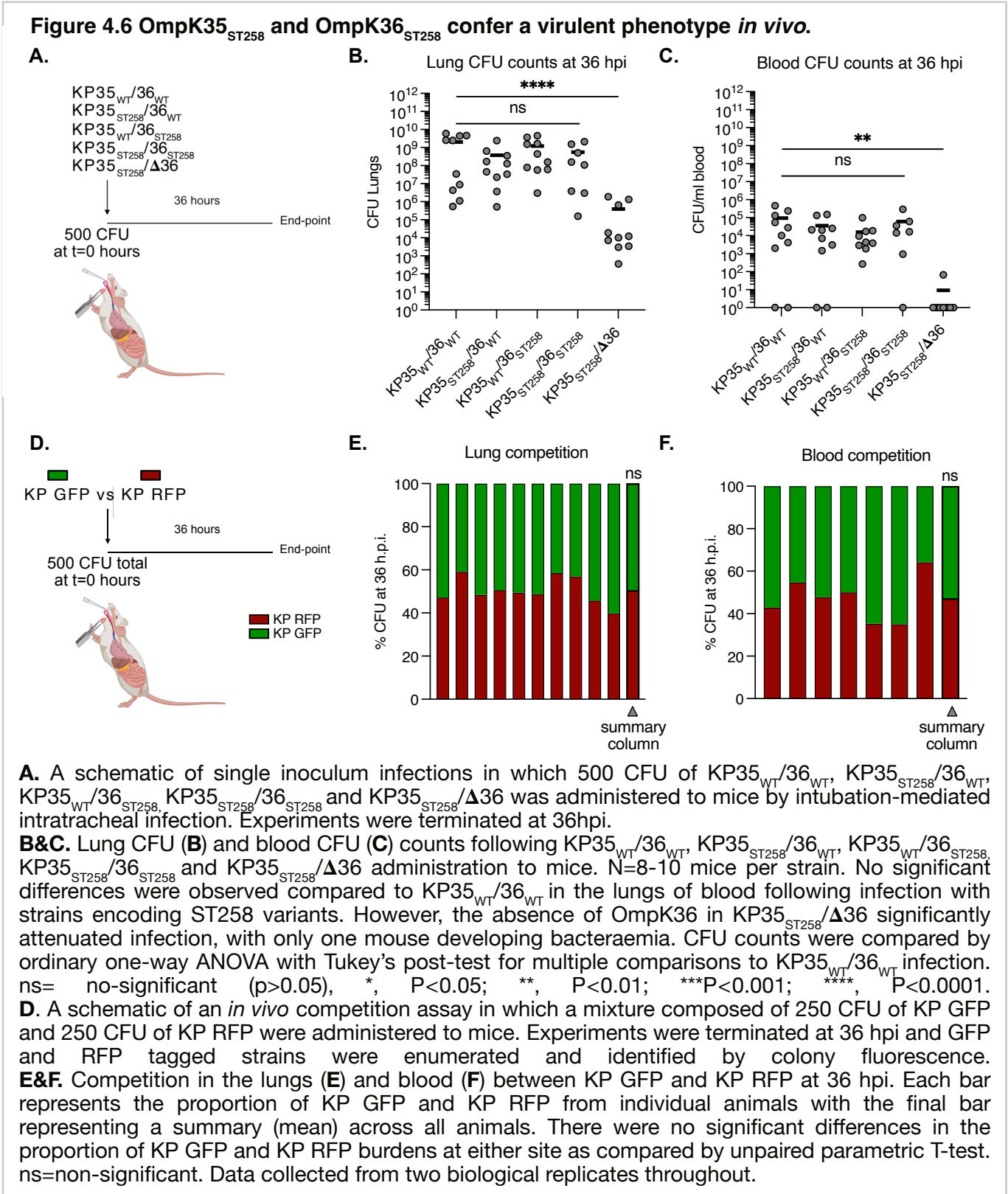
KP35_{ST258}/36_{ST258(R127A)}, indicating that there was no functional impact of the salt-bridge in augmenting resistance (**Figure 4.5B**). I further confirmed the absence of any change in MIC for ETP and IPM (**Figure 4.5B**).

The structural findings suggested that the L4 LSP insertion did not impact on pore diameter or the ability to form homotrimer complexes. As the L4 LSP insertion, along with the L3 GD insertion, were the major differences between OmpK36_{WT} and OmpK36_{ST258} sequences, I wanted to exclude it mediating a functional resistance phenotype. To that end I generated a strain in which the L4 LSP insertion was removed (KP35_{ST258}/36_{ST258ΔLSP}) and assessed MEM, ERT and IPM resistance. The deletion of the L4 LSP did not alter the level of resistance of any of the carbapenems tested, which remained high in both KP35_{ST258}/36_{ST258} and KP35_{ST258}/36_{ST258ΔLSP} (**Figure 4.5C**).

These findings indicated that L3 GD-insertion mediated OmpK36 pore constriction reduces meropenem diffusion driving the high MEM, ERT and IPM MICs conferred by OmpK36_{ST258}. Whilst present, the D114/R127 salt-bridge is not required to maintain a reduced pore radius. The L4 LSP motif is also present in OmpK36_{ST258} but does not impact on luminal diameter or pore functionality.

4.2.6. OmpK35_{ST258} and OmpK36_{ST258} confer a virulent phenotype *in vivo* associated with a competitive disadvantage

Having defined the OmpK35 and OmpK36 ST258 mutations and their impact on pore conformation I wanted to test their impact on virulence *in vivo*. I used our previously optimised preclinical model and proceeded to infect mice with 500 CFU of KP35_{WT}/36_{WT}, KP35_{ST258}/36_{WT}, KP35_{WT}/36_{ST258} and KP35_{WT}/36_{ST258}. I also generated an additional strain, in which OmpK36 was deleted (KP35_{ST258}/Δ36). As the *ompK35*_{ST258} allele results in a non-functional porin, KP35_{ST258}/Δ36 lacks both major KP outer membrane porins. A double OmpK35 and OmpK36 mutant has previously been demonstrated to be attenuated in the mouse, however, an intraperitoneal (i.p) infection route was used for this work¹⁸¹. Given respiratory infections by KP are the major site of human disease, and that intra-abdominal infections are usually polymicrobial in patients, we used this experiment to assess OmpK36 loss on virulence in the lung. At 36 hpi I assessed the pulmonary burdens of KP in the lung and dissemination to the blood and found that no WT or ST258 porin configurations impacted on virulence, with strains achieving high CFU counts at both sites (**Figure 4.6A-C**). No significant differences in the number of bacteria recovered from the lungs or blood were observed between infections with KP35_{WT}/36_{WT}, KP35_{ST258}/36_{WT}, KP35_{WT}/36_{ST258} or KP35_{ST258}/36_{ST258} strains (**Figure 4.6B&C**). However, when OmpK36 was absent in KP35_{ST258}/Δ36 the strain was highly attenuated, achieving low lung burdens with only one mouse (total n=10) developing a detectable KP bacteraemia (**Figure 4.6C**). These results indicated that the ST258 porin configuration



results in high virulence and that, whilst OmpK35 truncation has no effect on infection capacity, the presence of OmpK36 is required to achieve robust infection.

I wanted to more stringently assess the fitness cost associated with ST258 OmpK35 and OmpK36 isoforms *in vivo* and did this by competition assays in our mouse model. I had previously established the

glmS sfGFP (KP GFP) and *mRFP1* (KP RFP) tags in KP_{WT} did not result in an *in vivo* fitness cost (**Figure 3.6F**). I exploited these tags to identify bacteria at the end of the experimental protocol, by using UV transillumination and assessing the fluorescence of recovered colonies, before counting. I avoided using antibiotic selection markers to selectively plate out recovered bacteria as this would preclude us investigating the impact of the genes conferring these phenotypes in future work. I first assessed if the expression of either tag resulted in a fitness disadvantage, relative to each other, in our model and infected mice with a 50:50 mixture of KP GFP/KP RFP (250 CFU of each, total dose 500 CFU). In both these strains *ompK35*_{WT} and *ompK36*_{WT} were encoded on the genome. At 36 hpi mice I enumerated the number of KP GFP and KP RFP recovered from the lungs and blood and found that there were no significant differences between the numbers at either site (**Figure 4.6D-F**). This validated our approach to using these tags in our assay as the input and output ratio (1:1) of KP GFP and KP RFP was maintained.

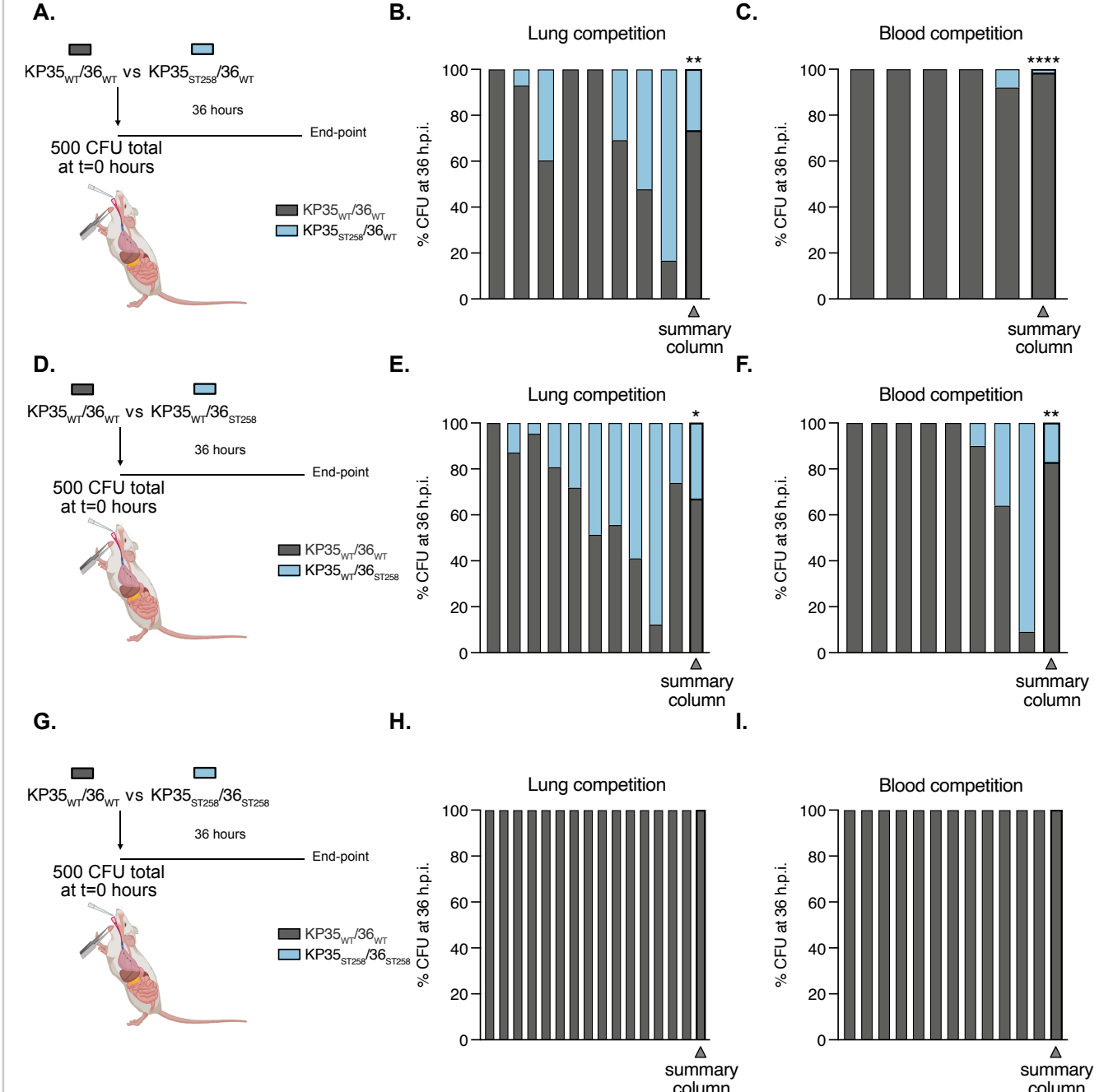
I started by evaluating the OmpK35 ST258 truncation in this assay and competed KP35_{WT}/36_{WT} against KP35_{ST258}/36_{WT} *in vivo*. I generated strains with both tags and in biological replicates reversed the fluorescent protein tag (i.e. replicate one KP35_{WT}/36_{WT} GFP vs KP35_{ST258}/36_{WT} RFP and replicate two KP35_{WT}/36_{WT} RFP vs KP35_{ST258}/36_{WT} GFP). I found statistically significant out competition of KP35_{ST258}/36_{WT} by KP35_{WT}/36_{WT} in the lungs of mice (**Figure 4.7A&B**). When I examined the proportion of KP35_{WT}/36_{WT} and KP35_{ST258}/36_{WT} in the blood of mice that became bacteraemic (5/8 mice) an even greater effect was observed, with only one animal having detectable KP35_{ST258}/36_{WT} in the bloodstream (**Figure 4.7C**). This indicated that the OmpK35_{ST258} truncation results in a competitive fitness defect.

I then proceeded to test the *in vivo* competition between KP35_{WT}/36_{ST258} and KP35_{WT}/36_{WT} (n=10 mice) to evaluate if either OmpK36_{ST258} or OmpK36_{WT} resulted in a fitness disadvantage. I found the same pattern observed in the OmpK35_{ST258} competition experiments with KP35_{WT}/36_{ST258} out competed by KP35_{WT}/36_{WT} in the lungs and the blood (**Figure 4.7D-F**).

Finally, as the clinical isolate KP_{ST258} had mutations in both OmpK35 and OmpK36 I asked what the impact of combined mutations would be, in competition with WT alleles. In this experiment I competed KP35_{ST258}/36_{ST258} against KP35_{WT}/36_{WT} and found complete out competition of KP35_{ST258}/36_{ST258} by KP35_{WT}/36_{WT} in the lungs (**Figure 4.7G&H**). I did not recover a single colony of KP35_{ST258}/36_{ST258} from the lungs of any infected animal (n=16) and in keeping with this, all colonies isolated from the blood were KP35_{WT}/36_{WT} (**Figure 4.7I**). This indicated that the combined OmpK35_{ST258} and OmpK36_{ST258} porin configuration results in a significant competitive disadvantage *in vivo*.

Taken together, in this series of *in vivo* experiments, I demonstrate that the expression of ST258 porins does not preclude achieving high levels of infection, with no differences observed when strains are administered as a single inoculum to experimental mice. However, when both OmpK35_{ST258} and

Figure 4.7 OmpK35_{ST258} and OmpK36_{ST258} expression results in a competitive disadvantage *in vivo*.



A. A schematic of an *in vivo* competition assay in which KP35_{WT}/36_{WT} was competed against KP35_{ST258}/36_{WT}. **B&C.** Competition in the lungs (**B**) and blood (**C**) between KP35_{WT}/36_{WT} and KP35_{ST258}/36_{WT} at 36 hpi. Each bar represents the proportion of KP35_{WT}/36_{WT} and KP35_{ST258}/36_{WT} from individual animals with the final bar representing a summary (mean) across all animals. KP35_{WT}/36_{WT} outcompetes KP35_{ST258}/36_{WT} at both sites. n=8 mice. Competition proportions were compared by unpaired parametric T-test. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. **D.** A schematic of an *in vivo* competition assay in which KP35_{WT}/36_{WT} was competed against KP35_{WT}/36_{ST258}. **E&F.** Competition in the lungs (**E**) and blood (**F**) between KP35_{WT}/36_{WT} and KP35_{WT}/36_{ST258} at 36 hpi. Each bar represents the proportion of KP35_{WT}/36_{WT} and KP35_{WT}/36_{ST258} from individual animals with the final bar representing a summary (mean competition) across all animals. KP35_{WT}/36_{WT} outcompetes KP35_{WT}/36_{ST258} at both sites. n=10 mice. Competition proportions were compared by unpaired parametric T-test. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. **G.** A schematic of an *in vivo* competition assay in which KP35_{WT}/36_{WT} was competed against KP35_{ST258}/36_{ST258}. **H&I.** Complete out competition in the lungs (**H**) and blood (**I**) between KP35_{WT}/36_{WT} and KP35_{ST258}/36_{ST258} at 36 hpi. Each bar represents the proportion of KP35_{WT}/36_{WT} and KP35_{ST258}/36_{ST258} from individual animals with the final bar representing a summary (mean competition) across all animals. KP35_{WT}/36_{WT} outcompetes KP35_{ST258}/36_{ST258} at both sites. n=16 mice. A T-test was unable to be performed as no KP35_{ST258}/36_{ST258} were recovered from either site in any animal. Data collected from two biological replicates throughout.

OmpK36_{ST258} are combined there is a significant competitive fitness disadvantage compared to OmpK35_{WT} and OmpK36_{WT} expression. The significant fitness cost of KP35_{ST258}/Δ36, evident when inoculated alone, demonstrates that these non-selective porins play key physiological roles in infection, as well as allowing carbapenems to traverse the outer membrane into the periplasm. OmpK35 also appears redundant, with a ubiquitous truncation in clinical isolates²³ and high infectivity by KP can be achieved, if OmpK36 is expressed in isolation.

4.2.7. The L3 Glycine-Aspartate insertion underpins the competitive disadvantage of OmpK36_{ST258}

I had generated several chimeric mutants in OmpK36 which allowed us to test what feature of OmpK36_{ST258} results in the observed competitive fitness disadvantage *in vivo*. Our current experiments were unable to determine whether the L3 GD, the L4 LSP or other variations in OmpK36_{ST258} underpinned this effect. I hypothesised that L3 GD-mediated pore constriction represented the most biologically plausible explanation and therefore, I started by exploring the L3 insertion. To test this, I competed KP35_{ST258}/36_{ST258} against KP35_{ST258}/36_{ST258ΔGD} (10 mice) and KP35_{ST258}/36_{WT+GD} against KP35_{ST258}/36_{WT} (10 mice) *in vivo*. I combined the data in these experiments and compared the number of bacteria recovered in the lungs from L3 GD insertion containing strains (KP35_{ST258}/36_{ST258} and KP35_{WT}/36_{WT+GD}) to those without the insertion (KP35_{ST258}/36_{ST258ΔGD} and KP35_{ST258}/36_{WT}) (**Figure 4.8A**). These *in vivo* competitions confirmed my hypothesis as strains encoding OmpK36 without L3 GD insertions outcompeted those containing this motif (**Figure 4.8B**).

Figure 4.8 legend continued.

D. Glucose liposomal swell assays with proteoliposomes reconstituted with either recombinant OmpK36_{WT} or OmpK36_{WT+GD}. Liposomes without protein (Empty) were used a control. Swelling occurs over the time course indicating that this low molecular weight sugar (180.156g/mol) is able diffuse across OmpK36_{WT} or OmpK36_{WT+GD} but not liposomes containing no protein.

E. The diffusion rate ($\Delta OD_{400nm}/\Delta t(s)$) of glucose calculated from data in **D**. No significant diffusion occurs in Empty liposomes with rapid diffusion occurring in both OmpK36_{WT} or OmpK36_{WT+GD} containing proteoliposomes. The rate of glucose diffusion ($\Delta OD_{400nm}/\Delta t(s)$) was compared by ordinary one-way ANOVA with Tukey's post-test for multiple comparisons between groups. ns=non-significant ($p>0.05$); *, $P<0.05$; **, $P<0.01$; *** $P<0.001$; ****, $P<0.0001$.

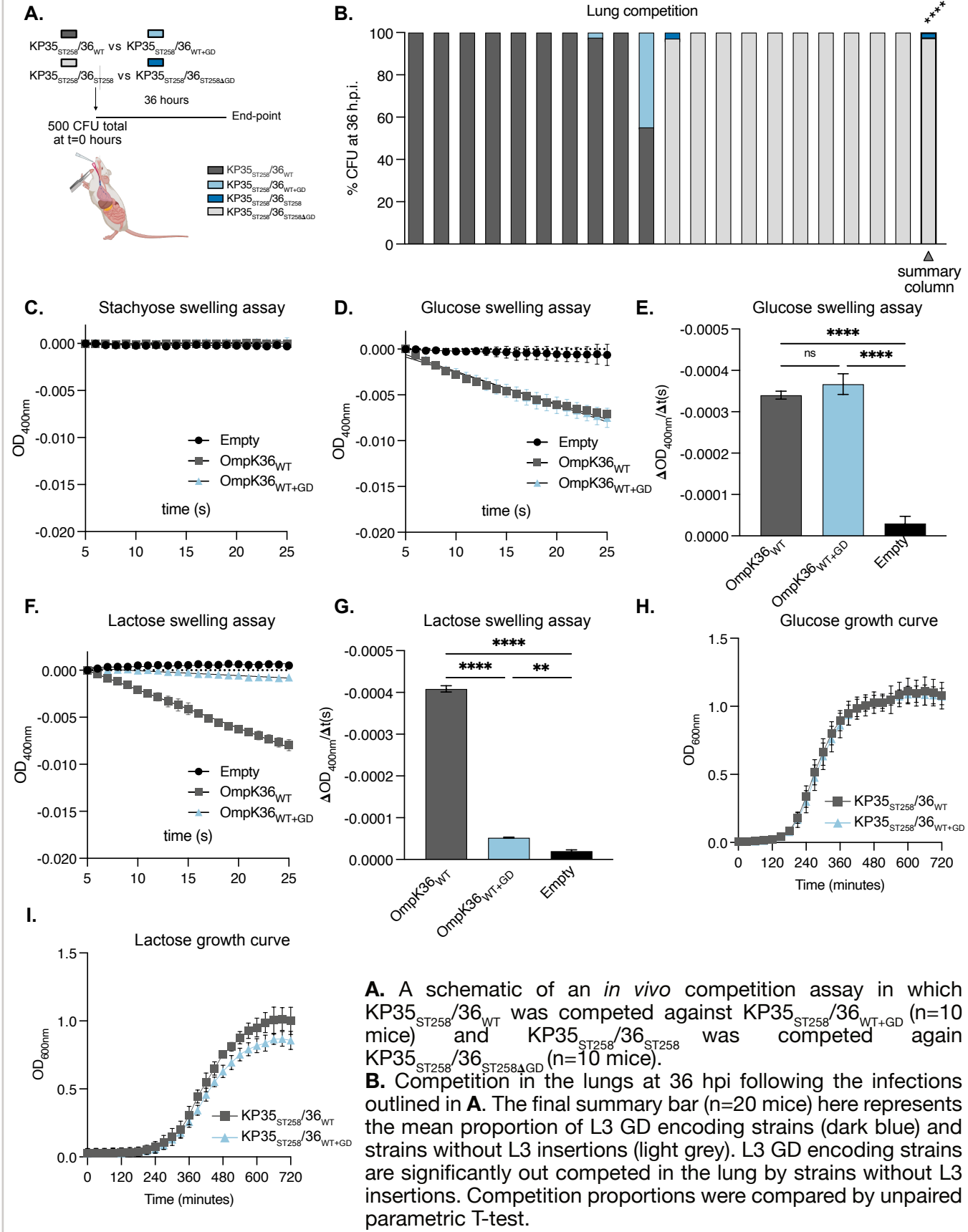
F. Lactose liposomal swell assays with proteoliposomes reconstituted with either recombinant OmpK36_{WT} or OmpK36_{WT+GD}. Liposomes without protein (Empty) were used a control. Significantly more liposomal swelling occurs in proteoliposomes containing OmpK36_{WT} compared to OmpK36_{WT+GD}.

G. The diffusion rate ($\Delta OD_{400nm}/\Delta t(s)$) of lactose calculated from data in **F**. No significant diffusion occurs in Empty liposomes. OmpK36_{WT} containing proteoliposomes have a significantly higher rate of lactose diffusion that those containing OmpK36_{WT+GD}. The rate of lactose diffusion ($\Delta OD_{400nm}/\Delta t(s)$) was compared by ordinary one-way ANOVA with Tukey's post-test for multiple comparisons between groups. ns=non-significant ($p>0.05$); *, $P<0.05$; **, $P<0.01$; *** $P<0.001$; ****, $P<0.0001$.

H. Growth curves in M9 media, containing 0.4% (w/v) of glucose as a carbon source, for KP35_{ST258}/36_{WT} and KP35_{ST258}/36_{WT+GD}. No differences in growth between the two strains were observed. Strains were grown in a plate reader at 37 °C and shaking at 200 rpm.

I. Growth curves in M9 media, containing 0.4% (w/v) of lactose as a carbon source, for KP35_{ST258}/36_{WT} and KP35_{ST258}/36_{WT+GD}. KP35_{ST258}/36_{WT+GD} achieves a lower growth rate and final OD_{600nm} under these conditions than KP35_{ST258}/36_{WT}. Strains were grown in a plate reader at 37 °C and shaking at 200 rpm. Data collected from three biological replicates throughout.

Figure 4.8 The L3 GD insertion underpins the competitive disadvantage conferred by OmpK36_{ST258}



A. A schematic of an *in vivo* competition assay in which KP35_{ST258}/36_{WT} was competed against KP35_{ST258}/36_{WT+GD} (n=10 mice) and KP35_{ST258}/36_{ST258} was competed against KP35_{ST258}/36_{ST258ΔGD} (n=10 mice).

B. Competition in the lungs at 36 hpi following the infections outlined in **A**. The final summary bar (n=20 mice) here represents the mean proportion of L3 GD encoding strains (dark blue) and strains without L3 insertions (light grey). L3 GD encoding strains are significantly out competed in the lung by strains without L3 insertions. Competition proportions were compared by unpaired parametric T-test.

C. Stachyose liposomal swell assays with proteoliposomes reconstituted with recombinant OmpK36_{WT} or OmpK36_{WT+GD}. Liposomes without protein (Empty) were used a control. No swelling occurs over the time course indicating that this high molecular weight sugar (666.579g/mol) is unable to diffuse across either OmpK36_{WT} or OmpK36_{WT+GD}.

I had previously shown that OmpK36 pore constriction results in a meropenem diffusion defect, but functionally, no growth defect in minimal M9 media, containing glucose as a carbon source, was evident (**Figure 4.1F**). As carbon sources are key for bacterial growth I asked if the influx of carbon sources, larger than the monosaccharide glucose, were hindered by L3 GD-mediated OmpK36 pore constriction. This was tested in two ways, biochemically in liposomal swelling assay and functionally by testing for growth defects in the presence of M9 media containing higher molecular weight sugars. The Beis laboratory assessed three carbohydrates; glucose (180.156g/mol), lactose (345.297g/mol) and stachyose (666.579g/mol), for diffusion across recombinant purified OmpK36_{WT} and OmpK36_{WT+GD}. Stachyose was unable to traverse either OmpK36_{WT} or OmpK36_{WT+GD}, with no liposomal swelling evident at the end of the assay (**Figure 4.8C**). Glucose was able to enter proteoliposomes containing OmpK36_{WT} and OmpK36_{WT+GD}, at statistically similar rates, but not empty control liposomes (**Figure 4.8D&E**). When the intermediate sized disaccharide, lactose, was assessed liposomal swelling only occurred in the presence of OmpK36_{WT}, with both OmpK36_{WT+GD} and empty liposomes demonstrating significantly smaller increases in size over the course of the assay (**Figure 4.8F&G**). I proceeded to test the growth of strains encoding these two isoforms (KP35_{ST258}/36_{WT} and KP35_{ST258}/36_{WT+GD}) for growth in M9 media with either 0.4% w/v glucose or 0.4% w/v lactose as the single carbon source (**Figure 4.8H&I**). KP35_{ST258}/36_{WT} and KP35_{ST258}/36_{WT+GD} had indistinguishable growth kinetics in M9 containing glucose, in keeping with the same rates of glucose diffusion observed by liposomal swelling assay. However, when KP35_{ST258}/36_{WT+GD} was grown in lactose containing M9 media a growth defect became evident in comparison to KP35_{ST258}/36_{WT}, achieving a lower overall OD_{600nm} at stationary phase and slower growth kinetics visible during log phase (**Figure 4.8I**).

In this final set of experiments, the L3 GD insertion is shown to be the molecular basis underpinning the competitive disadvantage *in vivo* of OmpK36_{ST258}. I suggest that one potential underlying cause for this defect is a reduced capacity for the diffusion of carbohydrate sources required for full virulence *in vivo*.

4.3. Discussion

In this results chapter I elucidate the precise molecular mechanisms by which alterations in OmpK35 and OmpK36 increase carbapenem resistance in an ST258 KP isolate. I chose to study the porins in this high-risk ST as it has been globally successful and is frequently associated with high meropenem MICs. In the case of OmpK35 I identified a frameshift mutation, resulting in a significant truncation and non-functional porin. Mechanistically, the increase in resistance is achieved by the absence of OmpK35 protein in the outer membrane that precludes antibiotic diffusion. OmpK35 is expressed at a lower level than OmpK36 and crystal structures indicate that it has a wider pore diameter, which would facilitate higher rates of diffusion, including higher molecular weight substrates (OmpK35 $3.6 \pm 1.4 \text{ \AA}$ vs OmpK36 $2.8 \pm 1.4 \text{ \AA}$ ⁶⁰). Despite the potential physiological advantages in facilitating the influx of more varied nutrients, OmpK35 appears to be redundant. In the *in vivo* infections KP35_{ST258}/36_{WT} was not attenuated when administered as a single inoculum in comparison to KP35_{WT}/36_{WT}, whereas the loss of OmpK36 in KP35_{ST258}/Δ36 significantly attenuated KP in this setting (**Figure 4.6B&C**). Whilst I was conducting this work a genomic analysis of 167 ST258 isolates from 20 countries collected over 17 years was published, which found the identical *ompK35* mutation to be ubiquitous in all these strains¹⁸². The findings suggest that this mutation occurred early in the expansion of the ST258 lineage and that OmpK35 truncation does not result in an attenuated phenotype in humans. This is in line with the findings from our translational mouse model⁶⁶. Two other groups have also investigated the role of OmpK35 in mouse models and demonstrated that loss does not attenuate KP infection. This work was conducted on different KP strain backgrounds, with one group infecting mice via the intraperitoneal route and the second by intranasal lung infection, adding further credence to my findings^{52,183}.

In the case of OmpK36 we found a more nuanced solution to increasing carbapenem resistance which took the form of luminal pore constriction. I propose that this has evolved as OmpK36 is required for virulence and truncation significantly attenuates KP *in vivo*. Whilst loss of OmpK36 would result in high levels of carbapenem resistance, as it is highly abundant in the OM, the accompanying fitness defect would preclude the strains carrying such mutations from clonally expanding. The underpinning mechanism leading to pore constriction was a glycine-aspartate (GD) di-amino acid insertion into L3 of the OmpK36 porin. The extended L3 conformation significantly reduces minimal pore diameter by 26% in OmpK36_{ST258} compared to OmpK36_{WT}. This abrogated carbapenem diffusion in liposomal swelling assays, whilst maintaining the ability to transport glucose. This also results in the formation of a supramolecular salt-bridge that stabilises the extended L3, by forming a bond with the opposing barrel wall. Although present, disrupting its formation did not alter the level of carbapenem resistance achieved, and therefore it plays no

role in further augmenting resistance. I also generated chimeric proteins to further validate our findings by inserting the L3 GD into OmpK36_{WT} (OmpK36_{WT+GD}) and deleting the L3 GD in OmpK36_{ST258} (OmpK36_{ST258ΔGD}). The finding that OmpK36_{WT+GD} replicated the resistance phenotype of OmpK36_{ST258} and that OmpK36_{ST258ΔGD} functionally behaved like OmpK36_{WT} allowed us to confidently attribute increased resistance to the GD insertion alone. When strains expressing OmpK36_{ST258} (KP35_{WT}/36_{ST258} and KP35_{ST258}/36_{ST258}) were administered to mice, as a single inoculum, no attenuation was observed *in vivo*, suggesting that, despite OmpK36 pore constriction, significant physiological OmpK36 functionality is maintained. At the time that I published our findings another group carried out similar *in vivo* experiments and found no attenuation in the context of OmpK36 containing a L3 GD insertion. A different KP background strain was used for these infections and inoculum delivered by intranasal infection¹⁷⁰. This strengthens our findings as it replicates the data in a different model system suggesting that GD-mediated pore constriction does not significantly impair KP virulence. However, when I tested this more stringently, in our competition assays, I found that the L3 GD insertion results in a competitive fitness cost. I hypothesise that the narrower luminal pore diameter does interfere with the uptake of certain nutrients or the efflux of toxic waste products and this underpins the highlighted fitness disadvantage. To that end, I tested diffusion of the disaccharide lactose and found significant reductions in diffusion across OmpK36_{WT+GD} compared to OmpK36_{WT} containing proteoliposomes, and a growth defect in minimal media containing only lactose as a carbon source. Whilst lactose may not be a primary carbon source available in the alveolar fluid, there is no literature delineating the carbohydrate sources present at this *in vivo* niche, which precluded me from making a more physiological appropriate choice of carbon source to test.

In this series of experiments, I demonstrate that OmpK36 is a key non-selective porin in the outer membrane of KP. It is required for virulence but also mediates carbapenem entry into the bacterial cell. Given its position at this intersection, evolution has selected for structural modifications that reduce carbapenem entry whilst maintaining virulence. I discovered this adaptation in a highly successful multidrug resistant ST258 strain and propose this adaptation has, in part, led to its global success. In our laboratory strain (KP_{WT}), despite the expression of the KPC-2 carbapenemase, a meropenem resistant MIC level was not achieved, suggesting that outer membrane porin changes are required in a two-step process to achieve robust carbapenem resistance. Since this work was published bioinformatic software has been created that can rapidly analyse KP genomes⁶⁶. *ompK35* and *ompK36* gene analysis is built into the analysis pipeline including the identification of truncations in either gene and the presence of L3 GD and TD encoding *ompk36* variants²². When this analysis was applied to 1717 KP genomes collected as part of a European structured survey¹⁴⁵, 60% of carbapenemase expressing strains were found to have

defects in either or both genes. In contrast, porin mutations were only observed in 19.9% of strains that did not encode a carbapenemase gene. This indicates the widespread nature of these mutations and suggests that they play important roles as they have been recurrently selected in clinical isolates responsible for carbapenem-resistant human disease. This work focussed on a single isolate from the ST258 clonal lineage as, at that time, this sequence type predominated globally. However, in areas where ST258 is already endemic other high risk STs (e.g. ST307) now also contribute to a significant proportion of CRKP disease. An in-depth understanding of OmpK35 and OmpK36 changes in these STs would be of significant interest, as more genomes are sequenced^{165,184}, to identify if the changes that I observed here are more generalisable across the KP phylogeny.

There is a well-recognised shortage of new antibiotics in the clinical development pipeline and an urgent need to develop novel agents^{185,186}. In the case of CRKP the need is particularly pressing as the rates of carbapenem resistance in KP increase across the globe, coupled with KP's prevalence as a nosocomial pathogen¹⁸⁷. I propose that data contained here can be used in this effort as part of rational drug design. Specifically, hydrophilic antibiotic entities would need to be able to traverse the narrowest pore (L3 GD pore constricted OmpK36) and should not rely on entry via high luminal diameter OmpK35, as it is redundant. Several new agents with anti-KPC KP activity have been licensed, which include novel beta-lactamase inhibitor combinations and the siderophore-cephalosporin cefiderocol¹⁸⁸. In the case of beta-lactamase inhibitors, these are all hydrophilic in nature and delineating the route by which they traverse the OM is key in anticipating future mutations that will result in resistance to these drugs. Bacterial adaptation in the form of mutant KPC-variants (e.g. KPC-33) have already been reported that confer resistance to avibactam, which is used in combination with ceftazidime, as a CRKP therapy¹⁸⁹. In the case of cefiderocol, this cephalosporin has two advantages; the first is that it relies on entry via siderophore receptors as an iron-binding catechol moiety is contained within its structure, the second is that it is intrinsically resistant to KPC-mediated hydrolysis¹⁹⁰. This obviates the requirement for entry via OmpK35 or OmpK36 but does raise the question as to whether siderophore receptor mutants may emerge during therapy. Animal models predict that such mutants would be attenuated in dissemination from the lungs via the blood to the liver and spleen and this may negatively select against their emergence¹⁹¹. As has been observed in OmpK36 from our ST258 isolate, evolution often finds an elegant solution to such paradigms. I hope that with the advent of widespread bacterial genomic sequencing, in combination with the *in vitro* and *in vivo* techniques outlined here, we may be able to better detect and predict their cost to human health.

Chapter 5. OmpK36 depletion mediated by an inhibitory RNA secondary structure.

5.1. Introduction

The number of publicly available KP genomes has rapidly increased over recent years with several notable collections now available. This includes those sequenced in the EuSCAPE study (1717 genomes)¹⁴⁵ and those compiled into the Pathogen Watch collection (16,537 genomes)¹⁹². In the EuSCAPE study both CRKP and carbapenem sensitive KP isolates were collected between 2013 and 2014 across Europe. Genetic analysis demonstrated that four clonal lineages constituted 69.9% of carbapenemase-positive isolates and defined these as high-risk clones. These were ST11, ST15, ST101 and ST258/512, where ST258 and ST512 are single-locus variants of each other²¹, and given this evolutionary relatedness, grouped into a single clonal lineage for analysis purposes. In the previous work I had focussed on a single isolate from the high-risk ST258 clonal group. I wanted to expand on this, to ensure that this isolate was representative of the high-risk ST258/512 group, and exploited large genome collections to describe the natural variation in *ompK36* and discover a new mechanism of resistance.

Three broad mechanisms of OmpK36-mediated resistance are known to exist; loss of OmpK36 and its complete absence in the outer membrane (OM), reduced or absent OmpK36 resulting from transcriptional silencing and structural modification by L3 insertion-mediated pore constriction. Whilst truncations and absent OM OmpK36 have been described these mutations are rarely reported in clinical isolates. When present, they usually appear during carbapenem therapy which results in subsequent treatment failure^{193,194}. Scattered reports have described the underlying genetic mechanism leading to this and include point mutations (introducing premature stop codons), frameshift mutations and small deletions (resulting in aberrant coding not compatible with pore formation and/or stop codons). Insertion sequences have also been observed to directly insert into the *ompK36* open reading frame (ORF)²³. Absent OmpK36 results in high carbapenem MICs as no pore is present to facilitate carbapenem diffusion into the periplasm. However, this is a blunt evolutionary tool as OmpK36 cannot fulfil its physiological role and, therefore, strains without OmpK36 are highly attenuated in our and other *in vivo* models^{52,66}.

Transcriptional silencing can occur due to direct disruption of the promoter in the 5' untranslated region (5'UTR) or, by interfering with upstream regulators that bind in this region to promote *ompK36* transcription. Promoter disruption has been described in a single centre study in which the IS5 mobile genetic element was shown to be present in the upstream *ompK36* region. This insertion resulted in either promoter disruption or complete promoter deletion¹⁷⁶. IS5 element insertion significantly increased doripenem resistance as compared to strains lacking this disruption (n=7/23 isolates). When IS5-mediated complete promoter deletion was encountered (n=2/7 IS5 insertions) the highest doripenem MICs were

observed. This study also reported OmpK36 L3 GD insertions (n=8/23 isolates) which increased the doripenem MIC, albeit to a lower extent than complete IS5-mediated promoter deletion. In this small single centre collection of isolates promoter disruption and L3-mediated pore constriction did not co-exist. In contrast to these naturally occurring *ompK36* locus mutations, *in vitro* evolution experiments have been conducted, in which regulatory mutants with higher cephalosporin or carbapenem MICs have been discovered. One example of such work identified a loss-of-function mutation in an *ompK36* transcriptional regulator. Here, the MarR-family transcriptional repressor KvrA was lost by the spontaneous deletion of a 1159 bp region spanning the *kvrA* open reading frame¹⁹⁵. OmpK35 and OmpK36 protein abundances were reduced 3-fold, in the context of this mutation, when assessed by liquid chromatography-tandem mass spectrometry. When a clean *kvrA* mutant was generated in a clinical isolate, that co-expressed KPC-3, the meropenem MIC was doubled (128mg/L to 256mg/L) and the presence of this mutation also conferred resistance to the newly licensed combination drug therapy meropenem/vaborbactam¹⁸⁸. In other work, a group generated a mutant in *hfq*, a gene that encodes an RNA-binding protein that facilitates mRNA/sRNA interactions¹⁹⁶. This RNA-binding protein was not found to specifically regulate *ompK36* as 897 other genes were transcriptionally modified in the absence of *hfq* expression. Several biological processes were impacted, including alterations in envelope protein composition, capsular polysaccharide abundance and resistance to exogenous stressors such as H₂O₂. *ompK36* transcripts were reduced by 3.3-fold, when assessed by qRT-PCR and OM abundance was reduced when assessed by SDS-PAGE. Whilst this group did not pursue the effects on carbapenem resistance they did demonstrate attenuation in an intraperitoneal mouse model with the Δhfq KP mutant. However, the global changes in the gene expression induced by *hfq* deletion precludes this *in vivo* fitness cost being specifically attributable to OmpK36 depletion. The prevalence and distribution of *kvrA* and *hfq* mutations remain unknown in clinical isolates and whilst they add to our understanding of OmpK36 regulation, their impact in clinical antimicrobial resistance is not clear.

Evolutionary mutations are selected in bacteria in response to changing environments and a strong selection pressure for nosocomial pathogens is anti-infective therapy¹⁹⁷. However, most mutations are likely to be deleterious and therefore, a trade-off exists between genetic fidelity (accurate DNA replication) and genetic innovation (that enables the generation of adaptive mutations with advantageous phenotypes). Mutation rates are usually determined by the balance of error introduction, as mutations during DNA replication and the detection and reversion of these errors by mismatch repair systems¹⁹⁸. In the case of single nucleotide polymorphisms, in which the reading frame is preserved, two classes of mutations can occur; synonymous mutations in which codon redundancy results in ‘silent’ mutations, as

the encoded amino acid remains unchanged, or non-synonymous mutations. In the latter, nucleotide transitions or transversions result in an amino acid substitution or the introduction of premature stop codons. In non-synonymous mutations a functional impact can be predicted due to the change in properties in amino acid residue within the encoded protein or truncation. If such mutations occur in genes encoding proteins of known function the implications can be predicted *a priori*. When they occur in orphan genes this can be used as the basis to explore the role that these loci play in bacterial physiology, by generating isogenic deletion and wild-type strains and testing for phenotypes. Whilst the enormous amount of genetic sequencing has allowed for these exploratory methods to become routine for non-synonymous mutations, in the case of ‘silent’ synonymous mutations these are often disregarded as no phenotype is predicted in the protein translation. In this chapter I explore a synonymous mutation in *ompK36* that occurs at position 25 of the ORF which would be predicted to have no functional effect on OmpK36, as the translated peptide sequence remains unchanged. However, I demonstrate that the mutation has occurred on multiple occasions in high-risk carbapenem resistant clonal lineages, suggesting a selective advantage. I show, for the first time, that a naturally occurring open reading frame synonymous mutation results in a marked phenotype, increasing meropenem resistance by reducing OmpK36 abundance. This is mediated by the formation of an inhibitory mRNA secondary structure and illustrates the broad mechanisms by which KP successfully adapts to prevailing selection pressures and underpins its success as a global contemporary antimicrobial resistance threat.

5.1.1. Specific aims

1. To identify novel resistance mutations in *ompK36* from large genome collections.
2. To define the precise molecular mechanism by which a synonymous mutation (25c>t mutation) results in an carbapenem resistant phenotype.
3. To assess the impact of OmpK36 depletion on virulence *in vivo*.

Figure 5.1 Frequency and phylogenetic distribution of *ompK36* variants in a curated collection of ST258/512 genomes.

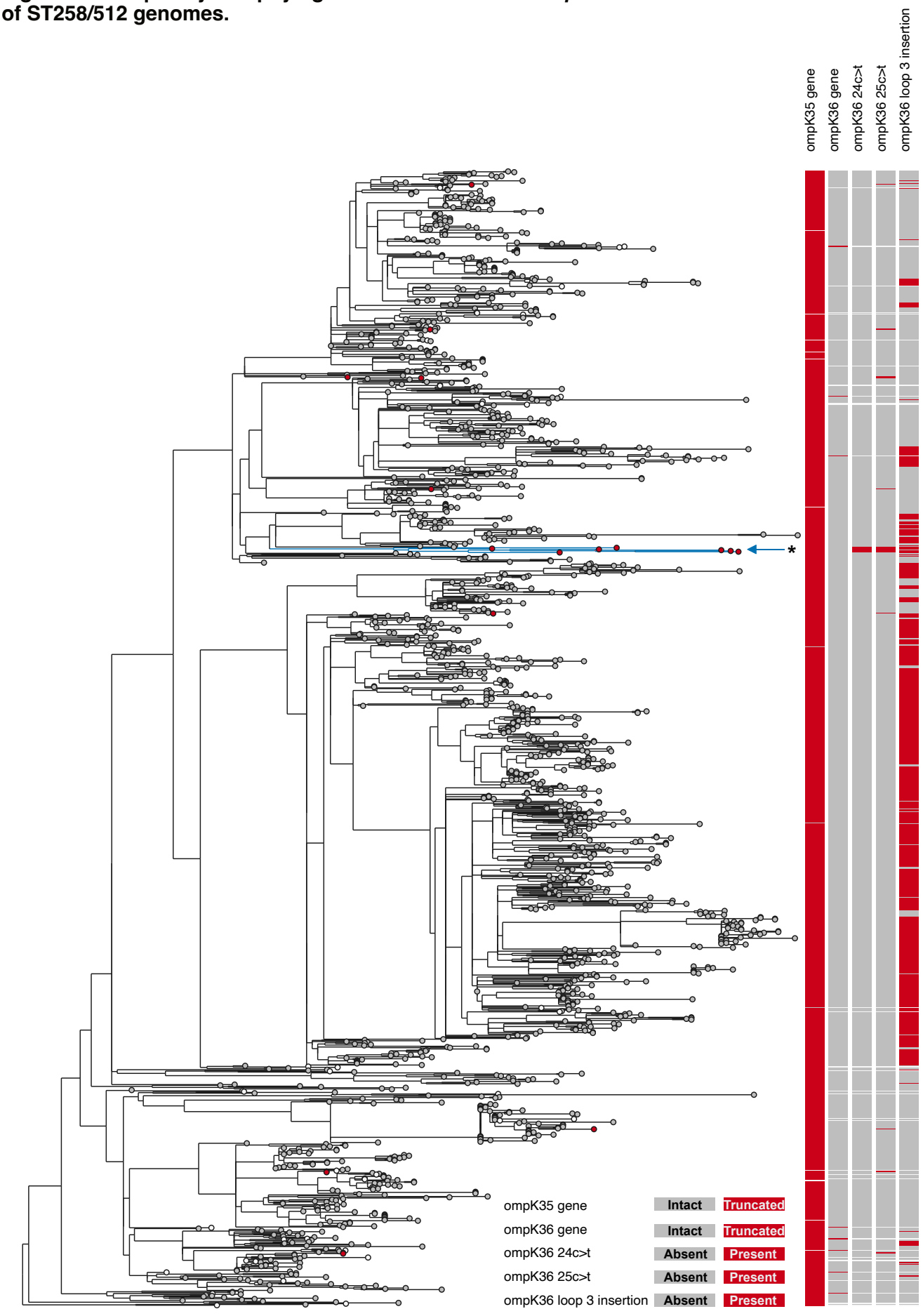


Figure 5.1 Frequency and phylogenetic distribution of *ompK36* variants in a curated collection of ST258/512 genomes. Phylogenetic tree of 1450 KP ST258/512 isolates constructed using vertically-inherited single nucleotide polymorphisms (SNPs). Data columns (left to right) show *ompK35* (intact/non-intact), *ompK36* (intact/non-intact), *ompK36* 24c>t mutation (present/absent), *ompK36* 25c>t mutation (present/absent) and *ompK36* L3 insertions (present/absent). A monophyletic cluster of *ompK36* variants encoding a 25c>t mutation is highlighted with an asterisk with branches coloured in blue.

5.2. Results

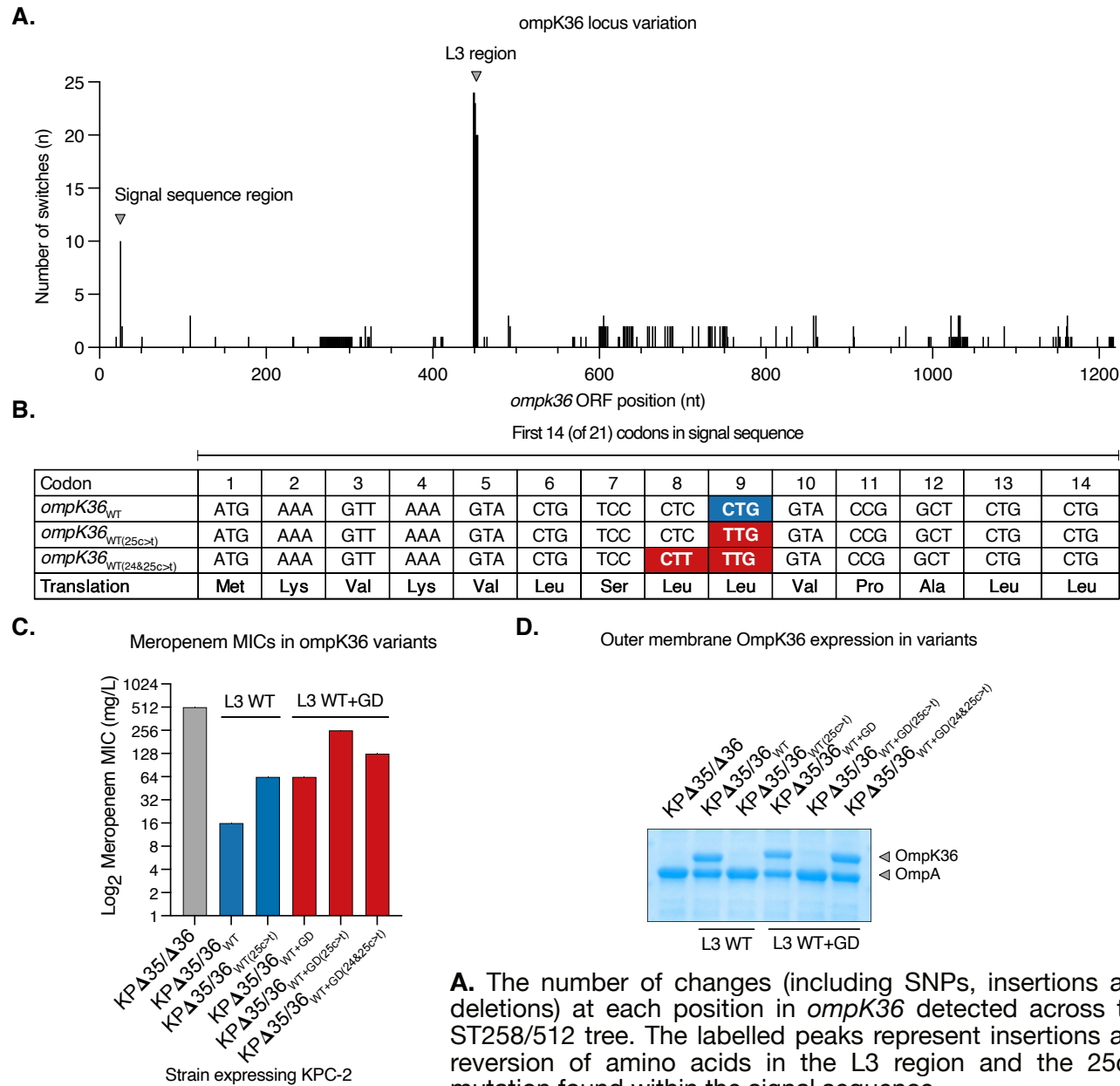
5.2.1. Identification and emergence of 25c>t mutation in *ompK36*

My previous work had established the role of OmpK36 L3 GD insertions in mediating pore constriction. This structural modification increased the carbapenem MIC but resulted in a competitive fitness cost *in vivo*. As I had studied the *ompK36*_{ST258} gene from a single isolate (KP_{ST258}) I wanted to evaluate mutations in *ompK36* from a larger collection of genomes. I started by focusing on ST258 and ST512 genomes and compiled a collection in collaboration with the Aanensen lab (Big Data Institute, University of Oxford) of 1450 publicly-available high quality genomes (ST258/512 collection). In keeping with the global success of ST258/512, the majority of genomes were derived from isolates in the Americas, Europe and the Middle East^{164,199–202}. The genomes were sequenced between 2003 to 2018, a period over which ST258/512 strains have disseminated globally, representing a major high-risk multidrug resistant clone^{23,145}.

Amongst the 1450 genomes the *ompK36* gene was unambiguously identified in 98.1% of isolates (1422/14550) by BLASTn (v.2.6.0)²⁰³. We used *ompK36*_{WT} as a query sequence and required ≥ 90% nucleotide similarity as identification criteria. Having solved the protein structure of OmpK36_{WT} we were able to define *a priori* a set the motifs required for functional pore formation. These were the presence of a start codon, a signal sequence (for periplasmic export), an L3 sequence, 16 β-barrels and a stop codon. We found that *ompK36* was infrequently lost by genetic truncation with 99.3% (1412/1422) of isolates encoding a functional pore. This was in keeping with our previous *in vivo* findings where KP35_{ST258}/Δ36 was highly attenuated suggesting that loss would preclude robust infection in humans. In 99.8% of the genomes *ompK35* was lost by truncation, in line with previous reported findings that had examined smaller ST258/512 genome collections²³.

We carried out a genomic analysis to study the evolution of the *ompK36* gene in this collection. To that end we constructed a phylogenetic tree (**Figure 5.1**) after removing recombined regions from genomes, to provide an accurate ancestral relationship between isolates. Variation in *ompK36* was mapped onto this phylogeny and changes in nucleotide positions predicted using a maximum parsimony approach. Overall the *ompK36* locus demonstrated a high level of conservation with 83.4% of positions unchanged. However, in two regions we observed a high number of base changes (**Figure 5.2A**). The greatest number of changes were observed in the L3 region and consisted of amino acid insertions (24 occurrences) that

Figure 5.2 The 25c>t mutation reduced OmpK36 protein abundance.



A. The number of changes (including SNPs, insertions and deletions) at each position in *ompK36* detected across the ST258/512 tree. The labelled peaks represent insertions and reversion of amino acids in the L3 region and the 25c>t mutation found within the signal sequence.

B. A schematic of the first 14 (of 21) codons in the signal sequence of *ompK36*. The synonymous 25c>t mutation occurs at codon position 9 (Leu9) resulting in a change of codon from CTG (*ompK36*_{WT}) to TTG (*ompK36*_{WT(25c>t)}). The 24c>t mutation occurs in position 8 (Leu8) resulting in a synonymous CTC to CTT codon switch.

C. Broth meropenem MICs of KPΔ35/Δ36, KPΔ35/36_{WT}, KPΔ35/36_{WT(25c>t)}, KPΔ35/36_{WT+GD}, KPΔ35/36_{WT+GD(25c>t)} and KPΔ35/36_{WT+GD(24&25c>t)}. The 25c>t transition increases the meropenem resistance achieved on both WT and WT+GD *ompK36* backgrounds. The additional 24c>t (WT+GD(24&25c>t)) mutation partially reverses the resistance achieved in the 25c>t transition. All strains express KPC-2, encoded on a pKpQIL-like plasmid.

D. Outer-membrane protein preparations, separated by SDS-PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis), followed by Coomassie staining from KPΔ35/Δ36, KPΔ35/36_{WT}, KPΔ35/36_{WT(25c>t)}, KPΔ35/36_{WT+GD}, KPΔ35/36_{WT+GD(25c>t)} and KPΔ35/36_{WT+GD(24&25c>t)}. The 25c>t mutation reduces OmpK36 expression conferred both WT and WT+GD *ompK36* backgrounds. The additional 24c>t in KPΔ35/36_{WT+GD(24&25c>t)} reverses the OM depletion of OmpK36 in KPΔ35/36_{WT+GD(25c>t)}. Representative image from two biological replicates.

included the di-amino acid GD insertion. We also observed the insertion of single amino-acids (Aspartate (D) and Asparagine (N)) and a further di-amino acid insertion, consisting of Threonine-Aspartate (TD) residues. Intriguing, together with insertions, we observed L3 insertion deletions (i.e. reversions to WT with loss of pore constriction). This is in keeping with our finding of a competitive *in vivo* fitness cost imposed by OmpK36 pore constriction and abrogation of substrate diffusion. We hypothesize that these reversions may be selected in the absence of antibiotic pressure to favour increased virulence and/or onward transmission.

The second region undergoing the highest level of evolution was within the N-terminal encoded Sec-dependent signal sequence (SS). Here, 10 occurrences of a c to t (c>t) transition occurred at position 25 (25c>t), however the role of this mutation was unclear as it was synonymous in nature (**Figure 5.2B**). In the 25c>t transition a leucine (leu) codon at position 9 is maintained (CTG (leu9) to TTG (leu9)), resulting in an identical amino acid SS translation. The high rate of convergent evolution suggested a selective pressure driving the recurrent emergence of this mutation, despite it lacking a predictable impact on phenotype. The phylogenetic appearance of the 25c>t was also unusual; whilst the mutation emerged at high frequency, the majority of isolates (11/18) occurred as singletons or pairs without evidence of onward transmission. In one case, a monophyletic clade (with onward transmission) of seven isolates was identified (**Figure 5.1**). These isolates were derived from different institutions in the USA between 2009-2013. On examining the SS of these isolates, an additional c>t transition at position 24 (24c>t) was identified. Furthermore, the 24c>t mutation is also a synonymous leucine mutation ((CTC (leu8) to CTT (leu8)) maintaining an identical SS translation (**Figure 5.1&5.2B**). Overall the prevalence of the 25c>t and combined 24&25c>t mutations was low at 0.7% (11/1422) and 0.5% 7(1422) (**Table 5.1**), respectively. However, given these mutations were synonymous and had emerged at high frequency, with divergent phylogenetic appearances (25c>t - no onward transmission without L3 insertions, 24&25c>t onward transmission with L3 insertions), we wanted to understand the role they played.

We started by expanding our analysis to *ompK36* genes in a more diverse (i.e. not restricted to ST258/512) and larger collection of KP isolates from a publicly available collection of 16, 086 KP genomes (Pathogen Watch collection)¹⁹². 92.6% (14888/16086) of the genomes had a functional (intact) *ompK36* gene. We identified the 25c>t mutation in 1.4% (215/14,888) of genomes and the combined 24&25c>t

Table 5.1 The frequency of 24c>t, 25c>t and combined 24&25c>t mutations in genomes

Mutation	ST258/512 collection		Pathogen Watch Collection	
	Frequency %	L3 insertion %	Frequency %	L3 insertion %
24c>t	0% (0/1422)	NA	0% (0/14888)	NA
25c>t	0.7% (11/1422)	0% (0/11)	1.4% (215/14888)	1.9% (4/215)
24&25c>t	0.5% (7/1422)	85.7% (6/7)	1.1% (161/14888)	93.2% (150/161)

Table 5.2 *Klebsiella pneumoniae* strains used in this chapter.

Strain name	<i>ompK35</i>	<i>ompK36</i> locus	Notes
KPΔ35/Δ36	Δ	Δ	Otherwise isogenic on KP _{WT} background
KPΔ35/36 _{WT}	Δ	<i>ompK36</i> _{WT}	Otherwise isogenic on KP _{WT} background
KPΔ35/36 _{WT(25c>t)}	Δ	<i>ompK36</i> _{WT(25c>t)}	Otherwise isogenic on KP _{WT} background
KPΔ35/36 _{WT+GD}	Δ	<i>ompK36</i> _{WT+GD}	Otherwise isogenic on KP _{WT} background
KPΔ35/36 _{WT+GD(25c>t)}	Δ	<i>ompK36</i> _{WT+GD(25c>t)}	Otherwise isogenic on KP _{WT} background
KPΔ35/36 _{WT+GD(24&25c>t)}	Δ	<i>ompK36</i> _{WT+GD(24&25c>t)}	Otherwise isogenic on KP _{WT} background
KPΔ35/36 _{WT(-14a>t)}	Δ	<i>ompK36</i> _{WT(-14a>t)}	Otherwise isogenic on KP _{WT} background
KPΔ35/36 _{WT(25c>t)(-14a>t)}	Δ	<i>ompK36</i> _{WT(25c>t)(-14a>t)}	Otherwise isogenic on KP _{WT} background

mutations in 1.1% (161/14,888) of isolates (**Table 5.1**). Whilst over half (51.3%) of the 25c>t mutations occurred in ST258/512 isolates, it was also present in other important multidrug resistant high risk STs including ST15 (19.9%), ST13 (4.5%) and ST11 (2.7%). We looked for and found no cases (0/14888) of a 24c>t mutation in *ompK36* in the absence of a 25c>t transition. This suggested that the 24c>t mutation occurs after the establishment of a 25c>t mutation. The diversity of the phylogeny encompassed in the Pathogen Watch collection precluded building a robust tree to examine onward transmission of these mutations.

We hypothesised that the selection pressure driving the emergence of the synonymous 25c>t and 24&25c>t mutation in *ompK36* was antibiotics, as the 25c>t was enriched in high-risk multidrug resistant STs. To that end, we examined their association with L3 insertions in *ompK36* genes, which we had demonstrated to be key in developing a robust carbapenem resistant phenotype (**Table 5.1**). In the larger Pathogen Watch collection we observed that L3 insertions were rarely found together with 25c>t mutations (1.9%, 4/215) but highly enriched in *ompK36* genes with the 24&25c>t mutation (93.2%, 150/161) (**Table 5.1**). In examining the ST258/512 collection the same pattern was observed, with no 25c>t transition containing *ompK36* genes encoding a L3 insertion but 85.7% (6/7) 24&25c>t mutants encoding a L3 GD insertion (**Table 5.1**).

In summary, we identified a frequently emerging 25c>t mutation in *ompK36*, that occurs independently of L3 insertions. The presence of the 25c>t appears to impair the successful onward transmission of ST258/512 KP isolates. An additional 24c>t mutation in *ompK36* was identified that only appears in combination with 25c>t mutations. Combined 24&25c>t mutations are associated with *ompK36* genes encoding L3 insertions with evidence of locoregional spread over a 4-year period (2009-2013), suggesting favourable onward transmission kinetics (**Figure 5.1**). The 25c>t encodes a synonymous leucine mutation at codon 9 (leu9) and the 24c>t mutation encodes a synonymous leucine mutation at codon 8 (leu8). I proceeded to investigate the role of these mutations in resistance and *in vivo* virulence and describe the mechanism by which these synonymous mutations result in a phenotype.

5.2.2. The 25c>t transition depletes outer membrane OmpK36 and is a carbapenem resistance mutation

I investigated the role of the 25c>t mutation by introducing the transition into our laboratory strain's (KP_{WT}) *ompK36* locus. I deleted *ompK35* (whole ORF deletion), as we had shown that it was lost in 99.8% of the ST258/512 collection (**Figure 5.1**), generating the strain KPΔ35/36_{WT(25c>t)}. All strains used in this chapter, including attributes, can be found in **Table 5.2**. KPC-2 carbapenemase was introduced by conjugation of the pKpQIL-UK plasmid. I started by assessing the meropenem MIC and compared it to strains lacking *ompK36* expression (KPΔ35/Δ36) and encoding *ompK36*_{WT} (KPΔ35/36_{WT}) lacking the 25c>t transition. The 25c>t transition in KPΔ35/36_{WT(25c>t)} increased the MIC 4-fold to 64mg/L from 16mg/L in KPΔ35/36_{WT}, which confirmed that the 25c>t transition is a resistance mutation (**Figure 5.2C**). I noted that the meropenem MIC in KPΔ35/36_{WT(25c>t)} remained lower than that achieved in the *ompK36* deletion mutant (512mg/L, KPΔ35/Δ36) indicating an intermediate resistance phenotype (**Figure 5.2C, Table 5.2**).

I then assessed the combined 24&25c>t mutation in *ompK36*_{WT} and, whilst I was able to genetically-engineer the mutagenesis vector, I was unable to introduce this into our laboratory strain's genome by recombination. This was of particular interest, as we had previously noted that the combined 24&25c>t mutation was rarely observed (0% in the ST258/512 collection and 1.9% in the Pathogen Watch collection) in *ompK36* sequences lacking L3 insertions (**Table 5.1**). I decided to simulate this situation, which had evolved in generations of clinical KP isolates, and generated the 24&25c>t transition on an *ompK36* L3 GD insertion encoding background. This genome substitution was tolerated and I was able to make the strain KPΔ35/36_{WT+GD(24&25c>t)}. In order to draw comparisons (as the L3 GD-insertion increases meropenem MIC by pore constriction) I also created the 25c>t on the L3 GD insertion background (KPΔ35/36_{WT+GD(25c>t)}) and as a control used the *ompK36*_{WT+GD} without any synonymous transitions present (KPΔ35/36_{WT+GD}). KPΔ35/36_{WT+GD} was found to have a meropenem MIC of 64mg/L, which represents the same increase in meropenem MIC that the 25c>t transition had on a WT L3 background (KPΔ35/36_{WT(25c>t)}) (**Figure 5.2C**). The 25c>t mutation further increased the meropenem MIC in the presence of the L3 GD to 256mg/L (KPΔ35/36_{WT+GD(25c>t)}) and the combined 24&25c>t mutations (KPΔ35/36_{WT+GD(24&25c>t)}) partially reversed this increase to an MIC of 128mg/L (**Figure 5.2C, Table 5.2**).

These results indicated a phenotypic effect of the synonymous transitions, with the 25c>t increasing meropenem MIC and the combined 24&25c>t partially restoring the phenotype to that of the WT signal sequence lacking any mutations. I suspected that these mutations may be playing a role in regulating OmpK36 expression levels, as the translated sequence was identical. I isolated OMP, separated them by SDS-PAGE and stained for protein by Coomassie (**Figure 5.2D**). In keeping with the meropenem MIC's the 25c>t mutation reduced the OmpK36 variant expression on both WT and WT+GD L3 backgrounds (**Figure**

Figure 5.3 A specific interaction between the 5'UTR and the 25c>t mutation underpins OmpK36 depletion.

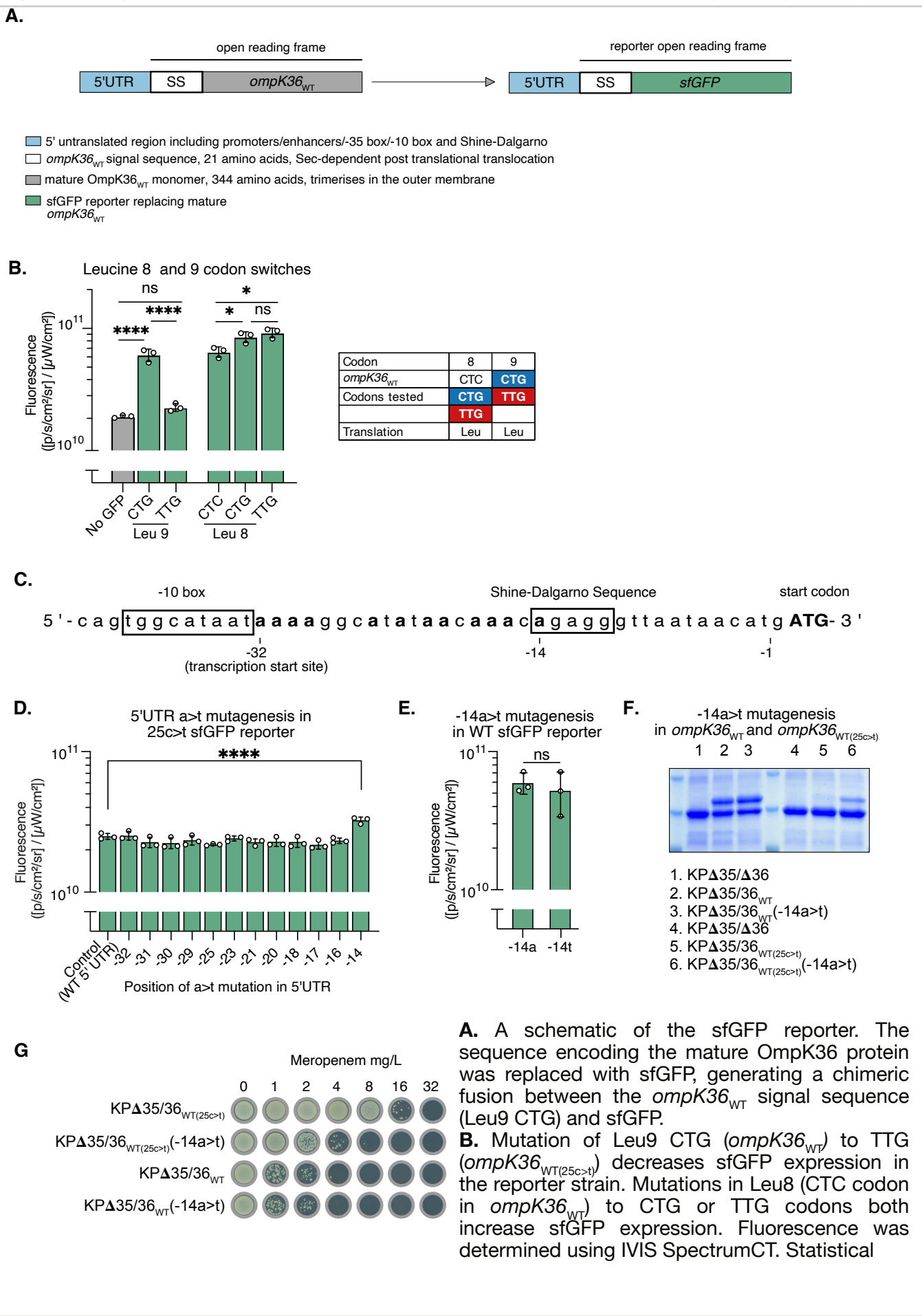


Figure 5.3 legend continued.

significance was determined by one-way ANOVA with Tukey's multiple comparison test. ns=non-significant ($p>0.05$); *, $P<0.05$; **, $P<0.01$; *** $P<0.001$; ****, $P<0.0001$. Data from 3 biological replicates.

C. Sequence of the *ompK36* 5'UTR. Adenine bases in the 5'UTR (in bold) were individually mutated to thymine. The ATG start codon is marked in bold. The -10 box and Shine-Dalgarno sequence are marked with boxes and labelled on the schematic.

D. Expression of the sfGFP in reporter strains encoding the *ompK36_{WT}* signal sequence is specifically increased upon mutation of the adenine in position -14 to thymine (-14a>t). The -14a marks the start of the Shine-Dalgarno sequence (SDS). Fluorescence was determined using IVIS SpectrumCT. Statistical analysis was performed by one-way ANOVA with Dunnett's multiple comparison post-test. *, $P<0.05$; **, $P<0.01$; *** $P<0.001$; ****, $P<0.0001$. Data from 3 biological replicates.

E. Mutation of the adenine in position -14 to thymine (-14a>t) in the reporter encoding the *ompK36_{WT}* signal sequence does not impact sfGFP expression. No significant difference was found following comparison by a Student's *t* test. ns=non-significant ($p>0.05$). Data from 3 biological replicates.

F. Outer-membrane protein preparations, separated by SDS-PAGE, followed by Coomassie staining from KPΔ35/Δ36, KPΔ35/ 36_{WT}, KPΔ35/ 36_{WT}(-14a>t) KPΔ35/Δ36 KPΔ35/36_{WT}(25c>t) and KPΔ35/36_{WT}(25c>t)(-14a>t). No change in OmpK36 abundance was observed between KPΔ35/ 36_{WT} and KPΔ35/ 36_{WT}(-14a>t), in keeping with the reporter data in **E**. The -14a>t mutation significantly increased OmpK36 abundance in the KPΔ35/36_{WT}(25c>t)(-14a>t) compared to KPΔ35/36_{WT}(25c>t) in keeping with the reporter data in **D**.

G. Meropenem agar dilution for KPΔ35/36_{WT}(25c>t), KPΔ35/36_{WT}(25c>t)(-14a>t), KPΔ35/ 36_{WT} and KPΔ35/ 36_{WT}(-14a>t). The -14a>t mutation in KPΔ35/36_{WT}(25c>t)(-14a>t) reduces meropenem resistance compared to KPΔ35/36_{WT}(25c>t) in keeping with the increase in OmpK36 expression observed in **F**. F and G are representative images from 2 biological replicates.

5.2D). The 24&25c>t mutation restored expression of OmpK36_{WT+GD} reversing the depletion induced when the 25c>t mutation was introduced alone (**Figure 5.2D**).

5.2.3. OmpK36 depletion is not due to codon bias in the 25c>t mutation

In the standard genetic code six codons (CTG, CTA, CTC, CTT, TTG and TTA) encode leucine and, together with arginine (CGG, CGA, CGC, CGT, AGG and AGA), this represents the highest observed codon redundancy. I wanted to investigate if the reduced expression of OmpK36 in the 25c>t mutation was related to the codon switch in leu9 from CTG to TTG. In *E. coli* codon bias is well described and, in the case of leucine, CTG codons are common (55% ORFs) and TTG codons less frequently utilised (11%)²⁰⁴. I started by performing an analysis of KP_{WT} and found a marked codon bias, with CTG and TTG leu codons used in 64.9% and 5.9% of ORFs, respectively (**Table 5.3**). In order to exclude that this leucine codon bias was specific to our KP strain, I analysed the ST258/512 collection (1450 genomes) and found the same bias; the range of CTG codons falling between 57.0% and 65.1% and TTG codons between 5.9% and 9.3% in all ORFs.

Table 5.3 The frequency of leucine codon usage in KP_{WT}.

	TTA	TTG	CTT	CTC	CTA	CTG
Raw Numbers (codons)	9807	10420	12178	24122	4529	112885
%s	5.6	6.0	7.0	13.9	2.6	64.9

I generated a fluorescent reporter strain, to evaluate OmpK36 expression *in vitro*, in which the structural component of the OmpK36 ORF was replaced with sfGFP. This reporter maintained both the upstream promoter and the N-terminal SS coding region, within which the 25c>t mutation is located (**Figure 5.3A**). I replaced the endogenous *ompK36* genome locus with this reporter and investigated the effect of codon bias by introducing codon switching mutations. I started by generating two fluorescent reporters in leu9, one in which the common CTG codon (representing *ompK36*_{WT}) was present and one encoding the rarely used TTG codon (representing *ompK36*_{WT(25c>t)}). When fluorescence was measured, I found high sfGFP signal in the presence of the CTG leu9 codon and significantly lower fluorescence in the TTG leu9 reporter (**Figure 5.3B**). A strain without sfGFP was used to control for autofluorescence and I found no significant difference in signal between this and the TTG leu9 reporter, indicating the induction of significant signal suppression (**Figure 5.3B**). These findings validated this reporter system's ability to assess the impact of SS mutations on OmpK36 expression, as they reproduced the findings I observed in meropenem MIC (**Figure 5.2C, Table 5.2**) and OM OmpK36 abundance (**Figure 5.2D**) observed between KPΔ35/36_{WT} (CTG leu9) and KPΔ35/36_{WT(25c>t)} (TTG leu9).

I then exploited the CTC encoding leucine at codon 8 (leu8) (**Figure 5.3B**) and generated further sfGFP fluorescent reporters. I changed the CTC to either the common CTG or rare TTG leucine codons. If the CTG to TTG codon switch underpinned the reduction of OmpK36 expression in leu9, I anticipated that a similar reduction in sfGFP signal would be expected in the leu8 position. However, both CTG and TTG leu8 codon switches significantly increased the sfGFP signal compared to the WT CTC leu8 codon (**Figure 5.3B**). This indicated that TTG codon switch, in itself, does not underpin OmpK36 depletion in the 25c>t mutation and that the mechanism of the resistance mutation was unrelated to codon bias.

5.2.4 A specific base interaction in the 25c>t *ompK36* mRNA transcript depletes OmpK36

Given the 25c>t mutation is synonymous and the Sec-dependent SS maintained this suggested that the reduced abundance of OmpK36 in the OM was not due to reduced periplasmic export. In the strain that I had engineered, KPΔ35/36_{WT(25c>t)}, the endogenous 5' UTR, including promoter and regulatory elements are maintained. I therefore excluded transcriptional changes underpinning OmpK36 downregulation, as the 25c>t mutation occurs in the ORF, and does not involve these sequences. Our leu8 CTC to CTG and TTG substitutions (**Figure 5.3B**) suggested that the codon switch, in itself, was not the underlying mechanism, but that the specific c>t base change, at position 25, was directly responsible for the observed phenotype. I examined the literature for potential mechanisms and found that in *E. coli*, intramolecular interactions could be engineered, in which post-transcriptional RNA secondary structures were induced, that blocked ribosomal binding to the Shine-Dalgarno sequence (SDS) (**Figure 5.3C**)²⁰⁴. This was shown to reduce the translation initiation and in turn reduce protein expression. Whilst such mutations could be

introduced throughout the ORF, they were more likely to occur in the 5' end of the gene, due to its proximity to the upstream SDS. However, these mutations were all experimentally induced and naturally occurring mutations of this type are not known to exist in natural bacterial populations. Nonetheless, I proceeded to test if the 25c>t mutation in *ompK36* was reducing protein expression in this manner and designed a series of *in vitro* mutagenesis experiments to test this hypothesis.

I started by using the sfGFP reporter that I had previously generated in our leu9 (TTG) mutagenesis experiment, encoding the 25c>t mutation. I had shown that this results in a low level of sfGFP fluorescence, not significantly higher than that of a non-sfGFP labelled control KP strain (**Figure 5.3B**). I analysed the *ompK36* 5'UTR using BPROM²⁰⁵ to identify the -10 box (-40 to -33) and the hypothetical transcription start site (-32) and thus *ompK36* mRNA would be predicted to start immediately after this motif (**Figure 5.3C**). I identified the SDS (-14 to -10) by consensus with other ORFs and position relative to *ompK36* start codon (**Figure 5.3C**). In the region between -32 and -14 we identified 12 adenine bases that I proposed could interact with the uracil, encoded by the 25c>t mutation, in the RNA gene transcript by Watson-Crick base pairing. I generated 12 mutants, by individually changing each adenine to a thymine (a>t), to disrupt any potential interaction driving an inhibitory RNA secondary structure. I observed no increase in fluorescence signal when a>t mutations were introduced between the -32 to -16 positions, however, in the -14a>t a significant increase in signal was detected (**Figure 5.3D**). This derepression of sfGFP fluorescence suggested that a specific interaction was occurring between the 25u and -14a, the latter marking the start of the SDS. I wanted to ensure that generalised SDS functionality, in the context of the -14a>t mutation, was not resulting in this signal increase and, as a control, generated the -14a>t mutation in the fluorescent reporter encoding the WT (25c) SS. I found no significant changes in fluorescence signal between the -14a and -14t construct, excluding a more generalised ability of ribosomes to bind and initiate translation from an a -14a encoding SDS (**Figure 5.3E**).

I then introduced the -14a>t mutations into the 5' UTR of *ompK36*_{WT} and *ompK36*_{WT(25c>t)} genes and tested OM OmpK36 abundance and meropenem resistance (by agar dilution) to assess if the changes I observed in our fluorescence assay translated into functional effects mediated by altered OmpK36 expression. In the case of the *ompK36*_{WT} no changes were observed in OmpK36 expression levels in either OM OmpK36 or meropenem MIC (KPΔ35/36_{WT} and KPΔ35/36_{WT}(-14a>t) (**Figure 5.3E**) in line with our fluorescent reporter findings. When the -14a was present in 5'UTR of *ompK36*_{WT(25c>t)} (KPΔ35/36_{WT(25c>t)}) OM OmpK36 levels were low with visibly increased levels achieved after the -14t mutation (KPΔ35/36_{WT(25c>t)}(-14a>t)) was introduced (KPΔ35/36_{WT(25c>t)}(-14a>t), **Figure 5.3F**). In keeping with the OMP abundance findings, I observed that the meropenem MIC dropped after introduction of the -14t in *ompK36*_{WT(25c>t)} as meropenem diffusion increased via increased porin expression (**Figure 5.3G**).

Figure 5.4 The 25c>t mutation sequesters the *ompK36* mRNA into a stem loop structure.

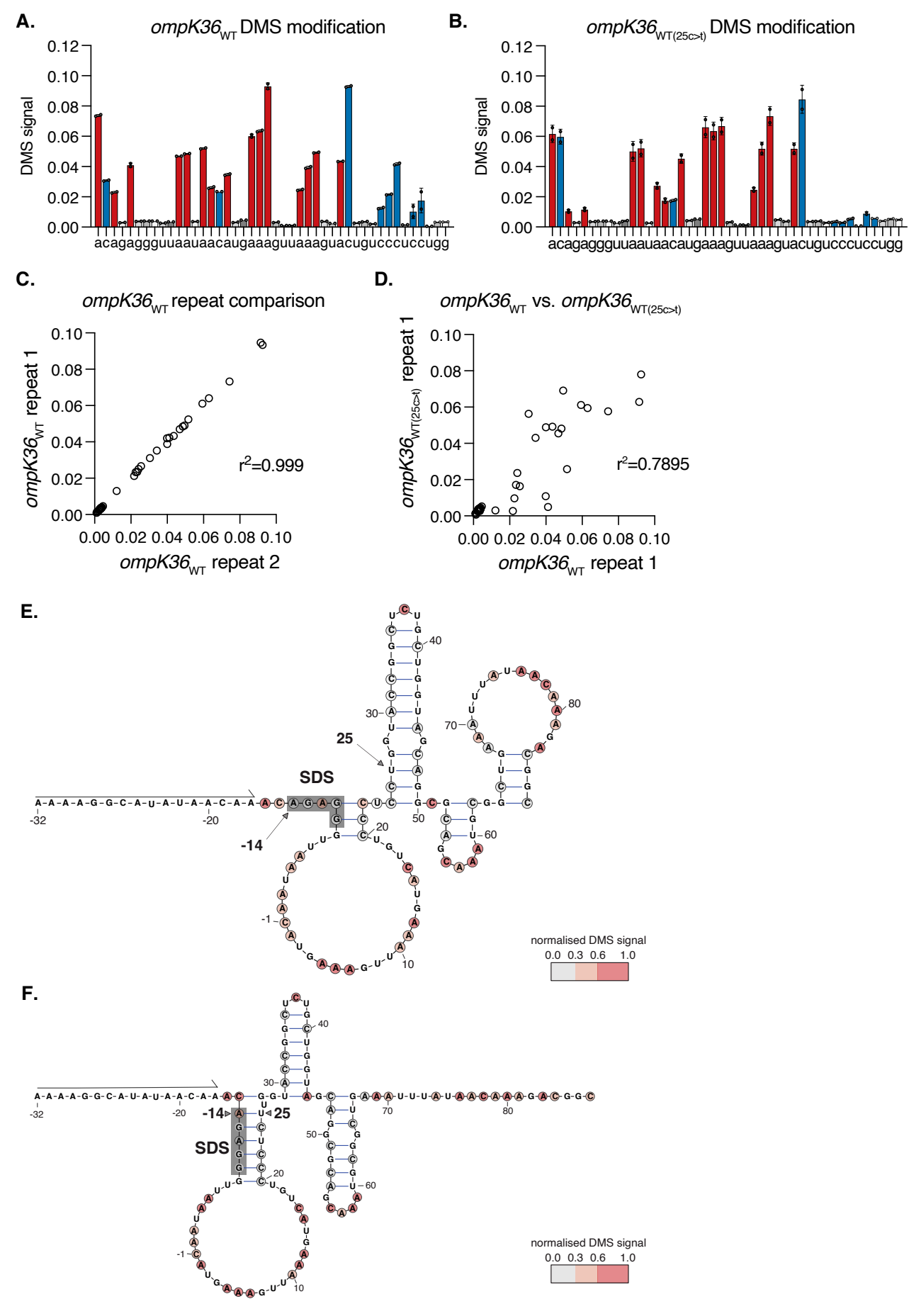


Figure 5.4 Legend.

A&B. Dimethyl sulphate (DMS) signal per nucleotide from full length *in vitro*-transcribed and refolded *ompK36* mRNA (*ompK36*_{WT} in **A** and *ompK36*_{WT(25c>t)} in **B**). Higher values correspond to increased base accessibility. Error bars=SD. Data from two biological replicates.

C&D. Comparison of DMS reactivities from nucleotides -16a through 28g between *ompK36*_{WT} biological replicates (**C**) and between *ompK36*_{WT} repeat 1 and *ompK36*_{WT(25c>t)} repeat 1 (**D**). Correlation was assessed by Spearman's rank correlation and the coefficient (r^2) inset on both graphs.

E. DMS-constrained structure models of the 5' end of *ompK36*_{WT}. The Shine-Dalgarno sequence (SDS) is highlighted in a grey box with nucleotides -14 and 25 indicated with arrows. The SDS is relatively free from secondary structure, except for the final 2 guanine nucleobases.

F. DMS-constrained structure models of the 5' end of *ompK36*_{WT(25c>t)}. The SDS is highlighted in a grey box with nucleotides -14 and 25 indicated with arrows. The SDS is completely sequestered into a stem loop and there is a direct interaction between the -14a and 25u as predicted in Figure 5.3 D.

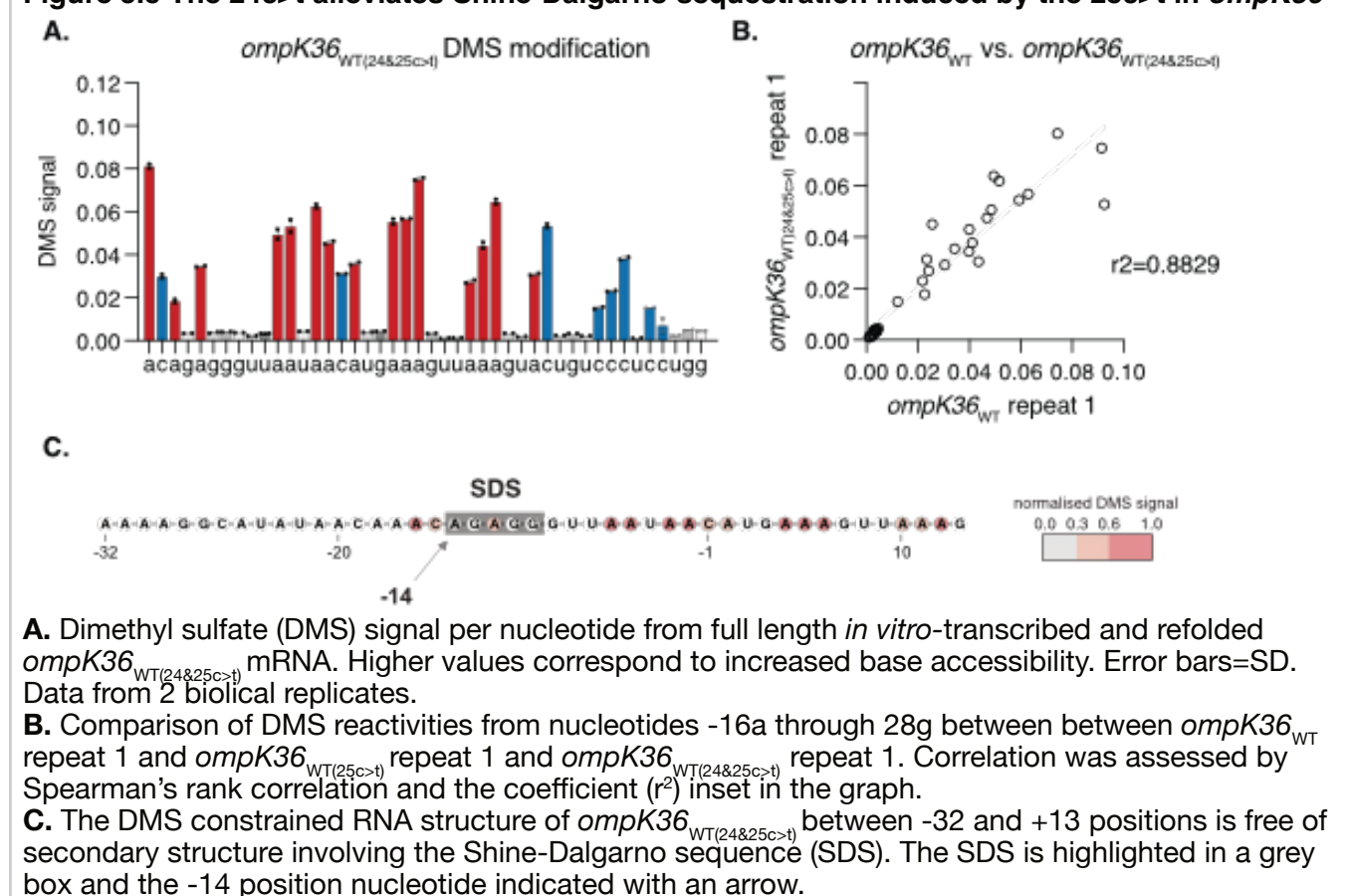
These data indicated that a specific RNA base interaction (-14a/25u) is the molecular basis in the 25c>t resistance mutation and this drives OmpK36 depletion.

5.2.5. The 25c>t *ompK36* mRNA structure sequesters the Shine-Dalgarno sequence into a stem loop

Whilst I had identified the specific RNA base interaction (-14a/25u) induced by the 25c>t *ompK36* mutation, I wanted to better understand the impact this had on RNA structure. In particular, as -14a marks the start of the SDS I wanted to know if this resulted in a significant conformational change, which could interfere with ribosomal binding hindering the initiation of translation. In the previous work, in which artificial inhibitory RNA secondary structures had been induced²⁰⁴ an *in silico* approach to RNA structural modelling had been adopted. I decided to use a more stringent method to determine the RNA structures of the *ompK36*_{WT} and *ompK36*_{WT(25c>t)} termed DMS-MaPseq²⁰⁶. In this technique, only free (i.e. not involved in RNA secondary structures) adenine and cytosine RNA nucleobases are modified by dimethyl sulphate (DMS) treatment, which leads to the introduction of mismatches during reverse-transcription (RT). The RT step uses the high-fidelity and efficient thermostable group II reverse transcriptase (TGIRT) and the pool of modified cDNA is subjected to next generation sequencing. The DMS signal (proportion of substitutions in the sequencing), resulting from the chemical modification and analysis, is then used to constrain an *in silico* model generated RNAfold²⁰⁷. In combining the *in silico* and *in vitro* data a more robust determination of RNA structure can be established.

I collaborated with the Rouskin laboratory (Harvard Medical School, Harvard University) to carry out DMS treatment and sequencing of modified cDNAs from *in vitro* transcribed *ompK36*_{WT} and *ompK36*_{WT(25c>t)}. This was carried out in duplicate, and we started by assessing the validity of the repeats. DMS signal was highly reproducible with only small deviations observed between repeats (**Figure 5.4A&B**). When we plotted the DMS signals (from bases -16 to +28) between duplicate runs for *ompK36*_{WT} we found a strong correlation ($r^2=0.999$), which implied the method was accurate and that a single RNA conformational structure was

Figure 5.5 The 24c>t alleviates Shine-Dalgarno sequestration induced by the 25c>t in *ompK36*



adopted (**Figure 5.4C**). We proceeded to compare the DMS signals between *ompK36*_{WT} and *ompK36*_{WT(25c>t)} and observed a weaker correlation ($r^2=0.7895$) implying that there was indeed a difference in RNA structure (**Figure 5.4D**). When we generated RNA structures by constraining the *in silico* models with these data for *ompK36*_{WT} and *ompK36*_{WT(25c>t)}, we observed significant differences, especially in the SDS region. In the *ompK36*_{WT} RNA the SDS is largely free from secondary structure, with only the final two 3' guanine nucleobases involved in a short stem structure (**Figure 5.4E**). However, in contrast, in the *ompK36*_{WT(25c>t)} RNA structure we observed that the entire SDS was sequestered into a long stem loop (**Figure 5.4F**). In addition, the *ompK36*_{WT(25c>t)} RNA structure also demonstrated a direct interaction between -14a and 25u that we had previously identified in our *in vitro* mutagenesis experiments.

Having established that the RNA structure and SDS involvement was significantly different between *ompK36*_{WT} and *ompK36*_{WT(25c>t)} we proceeded to solve the RNA structure for the *ompK36*_{WT(24&25c>t)}. We had previously shown that the second synonymous c>t mutation in the 24 position partially reversed the 25c>t mutation in terms of OM OmpK36 abundance and meropenem MIC (**Figure 5.2C&D, Table 5.2**) and was also a naturally occurring mutation in KP isolates. We wanted to know if the combined mutation alleviated the SDS sequestration that we had observed in the 25c>t, in line with our biochemical and resistance data. DMS treatment of *in vitro* transcribed *ompK36*_{WT(24&25c>t)} RNA between replicates was accurate with small differences observed between repeats (**Figure 5.5A**). In comparing the DMS signal between *ompK36*_{WT}

and *ompK36*_{WT(24&25c>t)} (-16 to +28) we observed a moderately strong correlation ($r^2=0.8829$) which was higher than previously calculated *ompK36*_{WT(25c>t)} correlation ($r^2=0.7895$), suggesting an overall structure more similar to the WT than the 25c>t (**Figure 5.5B**). We were unable to ascertain a complete RNA structure for *ompK36*_{WT(24&25c>t)} due to computational limitations in the *in silico* model. However, we were able to deduce, with accuracy, the structure between the -16 and +13 positions, which indicated a linear RNA, free from secondary structure (**Figure 5.5C**). This included the SDS, which was free from stem loops, predicting unhindered ribosomal access for translation initiation.

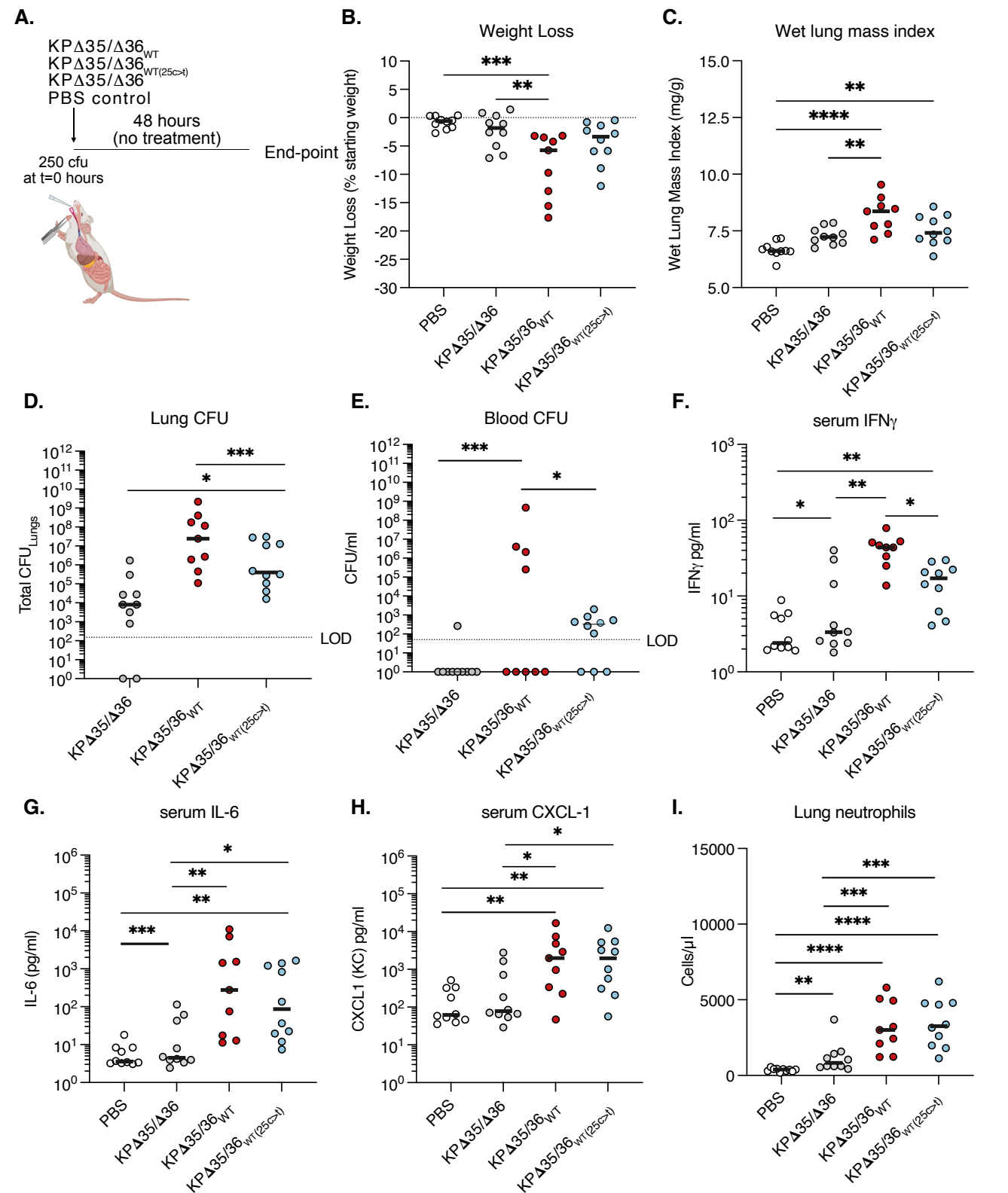
Taken together, the three RNA structures indicated that mechanistically the 25c>t mutation depletes OM OmpK36 by the induction of an RNA structure sequestering the SDS, impeding ribosomal access. The SDS is free of these interactions in the *ompK36*_{WT} and *ompK36*_{WT(24&25c>t)} allowing for translation initiation and high OmpK36 expression. To the best of our knowledge this is the first time that RNA structural changes have been implicated in any antimicrobial resistance mechanism.

5.2.6. 25c>t mediated outer membrane OmpK36 depletion attenuates KP *in vivo*

I had previously demonstrated that the loss of OmpK36, in KP35_{ST258}/Δ36, resulted in a significant attenuation *in vivo*. I now wanted to understand the impact of reduced OmpK36 expression, resulting from the 25c>t mutation, in our preclinical lung infection model. The previous experimental infections had focused on a short time course (36 hours) with a KP dose of 500 CFU administered to animals. I extended the time course of infection to 48 hours to allow us to introduce pharmacological interventions within the elongated time period. In order to achieve this, I decreased the inoculum size to 250 CFU, which resulted in the same severe phenotype at 48 hours post infection (hpi), that I had observed with 500 CFU at 36 hpi. In addition, I had focussed on bacterial burdens in the lungs and blood in our work on OmpK36 L3-GD insertions (Chapter 4) with no host derived metrics or physiological outcomes of infections that had been highlighted in our literature review (Chapter 3). I therefore integrated and assessed more outcome variables in the *in vivo* experiments. To that end, I optimised the data collection and measurement of weight loss, wet lungs mass index (a surrogate of acute lung injury), serum cytokine levels and lung neutrophil numbers as markers for the host's response to infection. I focussed on the pro-inflammatory cytokines IFN γ , IL-6 and CXCL-1 as others working in the field had demonstrated these to be raised following KP infection in mice.

I infected mice with 250 CFU of three isogenic strains of KP; KPΔ35/Δ36 (absent OM OmpK36), KPΔ35/36_{WT(25c>t)} (reduced OM OmpK36 abundance) and KPΔ35/36_{WT} (normal OM OmpK36 abundance). I also mock-infected one group with PBS to establish baseline parameters following sterile aspiration of an equal volume of fluid (as the inoculum suspension) into the lungs. I assessed weight loss by measuring the starting and final weight of animals and took a large volume of blood (for serum cytokine analysis and

Figure 5.6 The 25c> ompK36 mutation attenuates KP *in vivo*.



A. A schematic of single inoculum infections in which 250 CFU of KPΔ35/Δ36, KPΔ35/36_{WT}, and KPΔ35/36_{WT(25c>t)} were administered to mice by intubation-mediated intratracheal infection. Experiments were terminated at 48 hpi. A control group was inoculated with sterile PBS to provide baseline comparisons and to account for sterile aspiration of fluid into the lungs on outcome variables.

B. Animals infected with KPΔ35/36_{WT} show greater weight loss after 48 h compared to mock-infected animals and those infected with KPΔ35/Δ36. Weight loss in animals infected with KPΔ35/36_{WT(25c>t)} is not significantly different to that of the PBS or KPΔ35/Δ36 groups.

Figure 5.6 legend continued

C. Animals infected with KPΔ35/36_{WT} and KPΔ35/36_{WT(25c>t)} demonstrate significantly higher wet lung mass indices than uninfected (PBS control) animals. KPΔ35/Δ36 infection failed to induce a higher wet lung mass index than uninfected animals.

D&E. CFU counts in lungs (**D**) and blood (**E**), collected at 48 h post infection, revealed that infection with KPΔ35/Δ36 does not result in high lung bacterial burdens and bacteraemia. Infection with KPΔ35/36_{WT} results in high pulmonary bacterial burdens (**D**) and bacteraemia (**E**), while infection with KPΔ35/36_{WT(25c>t)} results in a trend towards lower lung CFU counts and reduced levels of bacteria in the blood.

F. Infection with KPΔ35/36_{WT} or KPΔ35/36_{WT(25c>t)} leads to increased serum IFN γ compared to mock-infected (PBS) animals. KPΔ35/36_{WT(25c>t)} infection results in significantly reduced serum IFN γ levels compared to KPΔ35/36_{WT} infection.

G,H& I. Infection with KPΔ35/Δ36, KPΔ35/36_{WT} and KPΔ35/36_{WT(25c>t)} induce significant increases in serum IL-6 (**G**), serum CXCL-1 (**H**) and lung neutrophils (**I**) compared to PBS treated controls. KPΔ35/36_{WT} and KPΔ35/36_{WT(25c>t)} induce higher levels of IL-6, CXCL-1 and lung neutrophils compared to KPΔ35/Δ36 infection.

n=9-10 mice per group for all infections. Graphs show median values. Statistical significance was determined by one-way ANOVA with Tukeys's multiple comparison post-test. *, P<0.05; **, P<0.01; ***P<0.001; ****, P<0.0001. Data throughout from 2 biological replicates.

bacteraemia assessment) by cardiac puncture under terminal anaesthesia. Mice were then humanely killed and lungs weighed to assess wet lung mass. Lungs were then homogenised to enumerate CFU and neutrophil counts contained in the tissue. Weight loss did not change significantly from baseline in mock-infected (PBS) mice (**Figure 5.6B**). KPΔ35/Δ36 infected animals lost little weight with no significant changes in weight compared to mock infected animals (**Figure 5.6B**). KPΔ35/36_{WT} infected animals lost substantial amounts of weight with significant differences observed between this group and both PBS treated and KPΔ35/Δ36 infected animals (**Figure 5.6B**). No significant weight changes were observed in KPΔ35/36_{WT(25c>t)} infected animals, however, there was a trend towards an intermediate phenotype between KPΔ35/Δ36 and KPΔ35/36_{WT} infected groups (**Figure 5.6B**). Acute lung injury, assessed by measuring wet lung mass index significantly increased in KPΔ35/36_{WT} and KPΔ35/36_{WT(25c>t)} compared to PBS treated animals (**Figure 5.6C**). However, in the absence of OmpK36 expression (KPΔ35/Δ36) no significant increases in wet lung mass index above mock-treated animals were observed (**Figure 5.6C**). Bacterial burdens achieved at 48 hpi in the lungs and blood were highest in KPΔ35/36_{WT} infections (**Figure 5.6D&E**). Significantly higher burdens of KPΔ35/36_{WT} and KPΔ35/36_{WT(25c>t)} were recovered in lung tissues compared to KPΔ35/Δ36 (**Figure 5.6D**). Whilst no significant differences were observed between KPΔ35/36_{WT} and KPΔ35/36_{WT(25c>t)} in the lungs, this did reach significance in dissemination to the blood (**Figure 5.6E**). The absence of OmpK36 in KPΔ35/Δ36 resulted in highly attenuated infection kinetics with low numbers of bacteria in the lungs and only 1 (n=10) mice achieving a detectable bacteraemia at 48 hpi (**Figure 5.6E**). The significant attenuation in KPΔ35/Δ36 infection was similar to that observed in the shorter time course with KP35_{ST258}/Δ36 in Chapter 4.

Serum IFN γ was significantly raised in KPΔ35/36_{WT} and KPΔ35/36_{WT(25c>t)}, but not KPΔ35/Δ36 infection compared to mock-treated animals (**Figure 5.6F**). I observed an intermediate phenotype in

KPΔ35/36_{WT(25c>t)} infection, with significantly reduced serum IFN γ levels compared to KPΔ35/36_{WT} infection (**Figure 5.6F**). The absence of OmpK36 in KPΔ35/Δ36 blunted the response with significantly reduced levels observed in comparison to KPΔ35/36_{WT} infection (**Figure 5.6F**). The serum IFN γ response was therefore graded and positively correlated with increasing amounts of OmpK36 in the OM of infecting KP strains.

IL-6 and CXCL-1 serum levels followed the same trends with KPΔ35/Δ36 failing to significantly induce increases over that observed in mock treated animals (**Figure 5.6G&H**). KPΔ35/36_{WT} and KPΔ35/36_{WT(25c>t)} infections resulted in high levels of both cytokines, with significant increases compared to infection with KPΔ35/Δ36 and PBS treatment (**Figure 5.6G&H**). This indicated that the reduced amounts of OM OmpK36 in the 25c>t mutation are sufficient to induce the same host response for these inflammatory mediators. Neutrophil counts in the lungs of infected animals increased with infection of any strain, irrespective of OmpK36 status, compared to uninfected animals (**Figure 5.6I**). However significantly higher numbers of neutrophils were found in KPΔ35/36_{WT} and KPΔ35/36_{WT(25c>t)} compared to KPΔ35/Δ36 infection (**Figure 5.6I**).

Taken together, these *in vivo* results indicated that the 25c>t mutation in *ompK36* results in attenuation, with an intermediate phenotype in bacterial burdens and the host's physiological response. This was in keeping with our observation that clonal expansion does not occur when the 25c>t *ompK36* mutation is present in clinical isolates, and that lower levels of infection may preclude onward transmission of strains bearing this mutation. These results also highlight the intersection that OmpK36 modifications play in mediating a carbapenem-resistant phenotype and virulence.

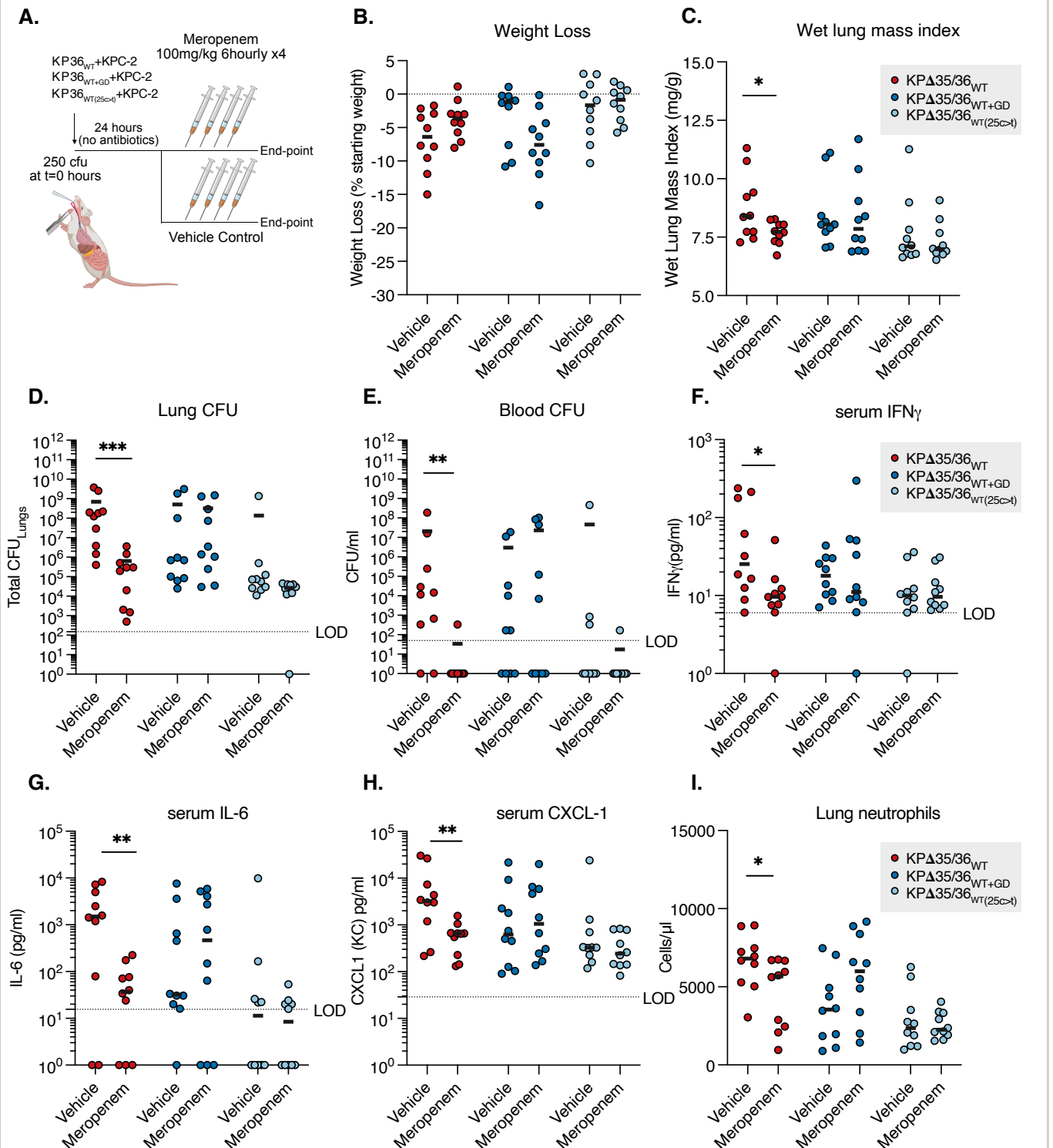
5.2.7. The 25c>t *ompK36* mutation is advantageous in the context of *in vivo* carbapenem therapy

Whilst this data had explained the phylogenetic appearance of the 25c>t *ompK36* mutation, with failed onward transmission, characterised by singletons/paired isolates in our ST258/512 tree (**Figure 5.1**) it failed to explain its recurrent emergence. I designed an *in vivo* experiment to evaluate if the increased level of carbapenem resistance translated into meropenem treatment failure. My working hypothesis was that if this were the case, recurrent emergences could be driven by the high selection pressure to achieve a more robust carbapenem resistant phenotype, within a patient, during meropenem therapy.

Figure 5.7 legend continued.

C-I. Meropenem treatment compared to vehicle control significantly reduced wet lung mass index (**C**), lung CFU counts (**D**), blood CFU counts (**E**), serum IFN γ (**F**), serum IL-6 (**G**), serum CXCL-1 (**H**) and lung neutrophil counts in the context of KPΔ35/36_{WT} but not KPΔ35/36_{WT+GD} or KPΔ35/36_{WT(25c>t)} infection. The statistical significance of the comparisons between vehicle and meropenem-treated animals was determined via a non-parametric Mann-Whitney test. *, P<0.05; **, P<0.01; ***P<0.001. Data throughout from 2 biological replicates.

Figure 5.7 L3 insertions and the 25c>t mutations in *ompK36* confer a fitness advantage in the context of meropenem therapy.



A. A schematic illustrating the experimental work flow in which 250 CFU (n=20 per group) of KPΔ35/36_{WT}, KPΔ35/36_{WT+GD}, KPΔ35/36_{WT(25c>t)} were administered to mice by intubation-mediated intratracheal infection. All strains expressed KPC-2, encoded on a pKpQIL-like plasmid. No treatment took place for 24 hours after which meropenem (n=10 per group) or vehicle (n=10 per group) were administered to mice every 6 hours until the experimental end-point at 48 hpi.

B. No significant changes in weight loss were observed in KPΔ35/36_{WT}, KPΔ35/36_{WT+GD}, KPΔ35/36_{WT(25c>t)} infection between animals treated with meropenem or vehicle control therapy.

I noted that the common L3 GD OmpK36 insertion resulted in the same meropenem MIC of 64mg/L as the 25c>t mutation (**Figure 5.2C and Table 5.2**). However, mechanistically I had demonstrated two divergent mechanisms for reducing meropenem diffusion across the OM; in L3 insertions a structural change abrogates diffusion but OM OmpK36 levels are maintained and in the 25c>t the pore remains structurally unchanged, but diffusion is limited by a reduction in absolute OmpK36 abundance. Therefore, in this final *in vivo* experiment I tested three strains, into which I had also introduced KPC-2 carbapenemase on a pKpQIL-UK background; KPΔ35/36_{WT} (lower meropenem MIC, normal OM OmpK36 abundance), KPΔ35/36_{WT(25c>t)} (high meropenem MIC, low OM OmpK36 abundance) and KPΔ35/36_{WT+GD} (high meropenem MIC, normal OM OmpK36 abundance). I infected 20 mice with each strain at the start of the infection time course, then, after 24 h without any interventions, randomised half the animals to receive meropenem (100mg/kg x 4 infections at 6 hourly intervals) or vehicle control (sterile water) by intraperitoneal (i.p.) injection. 3 h after the last injection I compared the outcome measures between meropenem and vehicle control treated animals (**Figure 5.7A**).

I observed no significant differences in weight loss between meropenem or vehicle control treated animals in KPΔ35/36_{WT}, KPΔ35/36_{WT(25c>t)} and KPΔ35/36_{WT+GD} infections (**Figure 5.7B**). However, in all other variables including acute lung injury (wet lung mass index), bacterial burdens (in the lungs and the blood), serum cytokine responses and lung neutrophils recruitment, MEM significantly impacted on the treatment of KPΔ35/36_{WT} compared to vehicle control (**Figure 5.7C-I**). This indicated that, in the context of OmpK36_{WT}, MEM treatment was effective, but in the context of KPΔ35/36_{WT(25c>t)} and KPΔ35/36_{WT+GD} infections no significant differences were observed between MEM and vehicle treatment, in any measured variable, indicative of treatment failure. I observed, as previously noted, that the pore constriction in KPΔ35/36_{WT+GD} resulted in robust infection, whilst OmpK36 depletion in KPΔ35/36_{WT(25c>t)} resulted in attenuated infection. However, both OmpK36 mutations resulted in MEM treatment failure, which may have driven their evolutionary emergence, in the face of this selection pressure. I postulate that weight loss was unchanged in the treatment KPΔ35/36_{WT} infection between MEM and vehicle as both therapies involved fluid administration, which remains a key therapy in the resuscitation of septic patients⁶.

5.3. Discussion

In this chapter I characterised *ompK36* genes, using large genome collections, and build on previous work that studied a single ST258 isolate. This demonstrated that *ompK36* is highly conserved and very rarely truncated in the high risk clonal lineage composed of 1450 ST258 and ST512 isolates. Whilst this is not a direct indication of OmpK36 essentiality it suggests its presence promotes successful infection and transmission in human infections. Additionally, the demonstration of the very high meropenem MIC in *ompK36* deletion strains provides further indirect supportive evidence of this hypothesis; if carbapenem resistance, in isolation, drives evolutionary success then these truncations would be anticipated to occur at higher frequency than the 0.3% prevalence found in this collection.

I initially conducted this analysis to estimate the emergence and prevalence of *ompK36* L3 insertions, as I had demonstrated them to result in a high carbapenem MIC, with a fitness cost only evident during *in vivo* competition. Indeed, I found the L3 region of *ompK36* underwent the highest number of changes with the emergence of multiple different amino acid insertions (D, N and TD) together with the GD insertion that I had studied in detail. The recurrent emergence of the 25c>t mutation in this collection suggested that this synonymous change may be advantageous. Indeed, I show that it is a resistance mutation that depletes OmpK36 from the OM. Importantly, I demonstrate it is present outside of the ST258/512 phylogeny, by using a more diverse and much larger genome collection¹⁹², indicating that the mutation is not restricted to a single sequence type.

There is a vast literature examining codon bias which has largely excluded rare codon usage in determining protein expression²⁰⁸. This system is complex and there are several biologically plausible reasons as to why codon bias may influence protein translation rates. These include the relative abundance of codons in ORFs, the number of tRNAs available with appropriate anticodon binding combined with the dynamics of tRNA charging²⁰⁹. The latter refers to the process by which uncharged tRNA (i.e those which have already been used in translation and are de-aminoacylated) are recharged by aminoacyl-tRNA synthetases, which add the correct amino acids to their corresponding tRNA species. I wanted to exclude this as an underlying cause for OmpK36 depletion as leucine has the highest degeneracy in the genetic code, with 6 possible codons and thus, has not been explored in detail. The experiments in which I change the leu8 codon from a CTC to CTG or TTG codon demonstrate that codon bias does not underpin OmpK36 depletion, as both CTG and TTG mutants increased expression in our fluorescent reporter system. On one hand this adds support to the idea that synonymous mutations can be silent, however, as I had demonstrated a clear phenotype (in the leu9 position) it suggests that recurrent mutations of this kind should be followed up with *in vitro* assessment. In the case of an important

gene with known function, such as *ompK36*, this may seem clear, however should such mutations occur in orphan genes they may be overlooked. This stresses the importance of genome collections with accompanying phenotypic data so that associations can be sought. However, such collections are not common for KP and require a large number of isolates to statistically explore these mutations. In the case of the EuSCAPE genome collection, meropenem MIC is available for each isolate which provides a highly valuable resource for such work²⁴. However, when considering this collection, only phenotypic data relating to antimicrobial resistance is available and it is not clear if this were to be expanded what phenotypes should be examined. Phenotypic assays would also need to be high-throughput, given the large number of isolates and whilst it would be interesting to test all 1717 isolates in our *in vivo* model, the high number of animals required would make this infeasible. This is especially true if we consider that not all clinical isolates appear to infect mice which would preclude us from obtaining meaningful phenotypic data. Our use of isogenic strains allowed us to study the *ompK36* 25c>t mutation on an otherwise identical background and attribute changes directly to this single base change in the genome.

This data builds on previous observations that artificially induced RNA secondary structures can modify protein expression²⁰⁴. Furthermore, it does so in clinical isolates and in the context of an important antimicrobial resistance phenotype. I show this in two ways, in the first I carry out rational mutagenesis of the 5' UTR of the *ompK36* gene. The systematic a>t substitutions, from the transcription start site to the Shine-Dalgarno sequence of *ompK36*, identified the specific interaction site that occurred only in the 25c>t *ompK36* ORF mutation. Importantly, I excluded that the -14a>t mutation results in generalised changes in Shine-Dalgarno functionality and ribosome binding ability; no differences in either GFP reporter fluorescence, OmpK36 abundance or meropenem resistance occurred when this 5' UTR mutation was introduced into the wild-type *ompK36* ORF lacking the 25c>t mutation. Furthermore, in the second approach, I collaborated to solve the structure of the 25c>t *ompK36* mRNA, which indicated both that this specific interaction was present but that it also sequestered the Shine-Dalgarno sequence into an extended stem loop. These complementary approaches support the hypothesis that this naturally occurring mutation depletes OmpK36 by inducing an inhibitory RNA secondary structure that impedes ribosome binding and OmpK36 translation initiation. The 24c>t mutation reverses the effect of the 25c>t on mRNA structure and increases the level of OmpK36 in the OM. This second mutation is interesting as it was not observed independently of the 25c>t and largely encountered in *ompK36* genes containing pore constricting L3 insertions. My interpretation of this finding is that a spontaneous mutation at the 25c>t position is mitigated by an additional 24c>t mutation, as reduced carbapenem diffusion has already been achieved by pore constriction.

In the final series of *in vivo* infections I link the phylogenetic appearances of the 25c>t *ompK36* mutation with experimental outputs in the translational model. I demonstrate that the absence of OmpK36 results in severe KP attenuation *in vivo* and extend the model outcomes assessed in Chapter 4. I include weight loss, acute lung injury, serum cytokine levels and lung neutrophil counts into our host assessment and find that the 25c>t mutation induces an intermediate phenotype. I demonstrate this in terms of both bacterial replication and bacteraemia, and in host-derived metrics. In particular, IFN γ responses, were graded according to the OmpK36 abundance in the infecting strain. This cytokine has been demonstrated to play an important role in KP infection and IFN γ ^{-/-} mice have increased susceptibility and poorer outcomes in KP lung infection²¹⁰. It is interesting to note that if I had focussed on only one aspect of infection, for example lung neutrophil numbers, that no differences would be observed between infecting strains, which illustrates the importance of multimodal assessment in animal models. I only carried out pulmonary infections in this work as time precluded the development of other models. However, given gut colonisation and subsequent invasive KP infection are associated in human studies²¹¹ it suggests that a gut model would be extremely helpful in understanding how KP has become so successful. Our laboratory strain is a hvKP strain and these are known to disseminate from the gastrointestinal tract and result in atypical infections, such as those in the central nervous system and hepatic abscesses⁹. ST258/512 strains fall into the classical KP phenotype therefore oral administration results in mice may be uninterpretable, due to pathotype specific features. Nevertheless, it would be useful to understand if OmpK36 truncations, L3 insertion mutants and the 25c>t mutation reduce KP levels at this key reservoir site in humans.

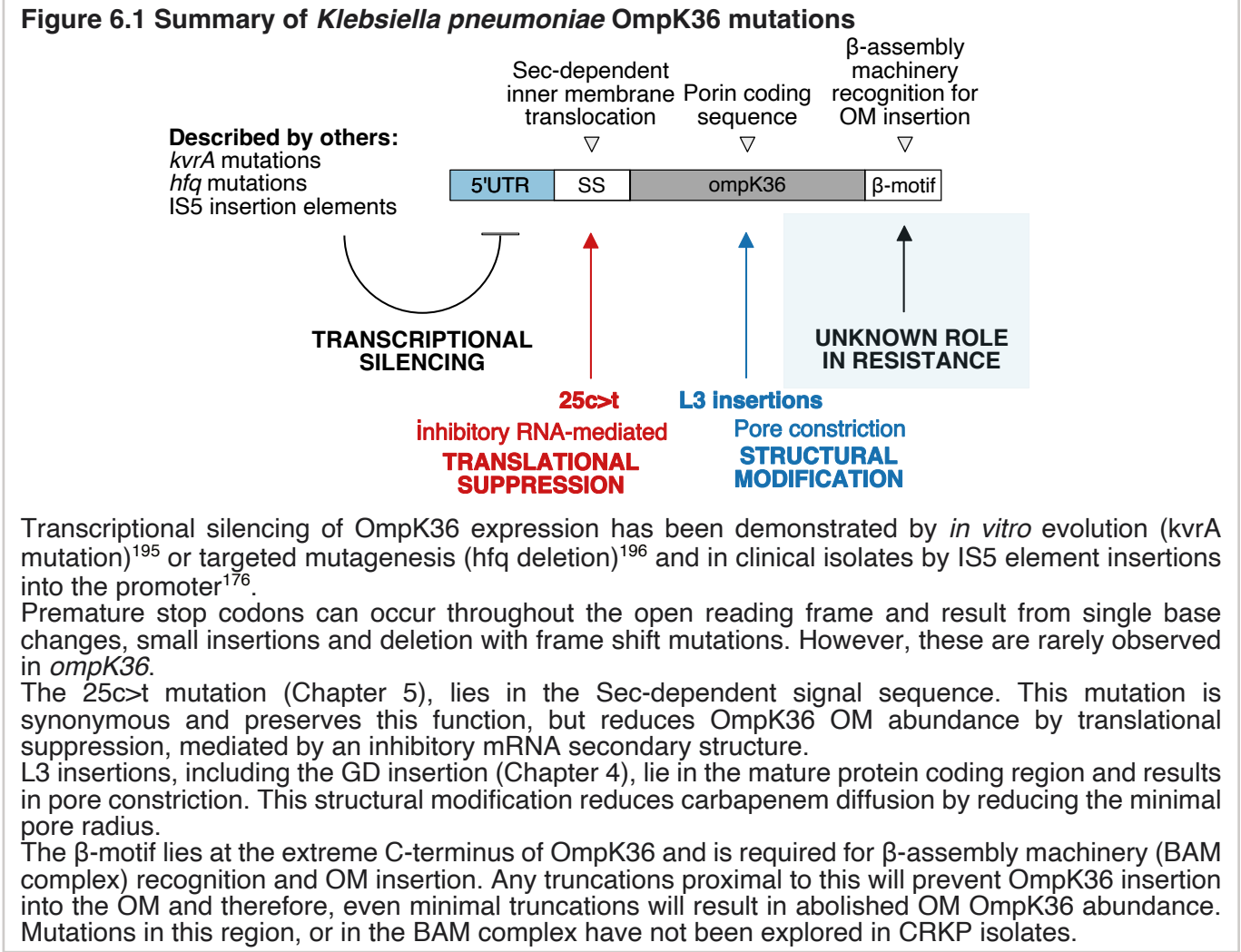
Finally, I used meropenem administration in mice to explain the recurrent emergence of the 25c>t mutation and to explore the potential advantage conferred by the L3 GD insertion. In these experiments OmpK36_{WT}-expressing strain infection could be successfully treated by meropenem administration. This was demonstrated in every outcome of infection apart from weight loss and I propose that this was due to fluid resuscitation of animals in both meropenem and vehicle control treated animals. The 25c>t mutation resulted in lower overall levels of infection but there was no difference with or without meropenem administration. It is this selective advantage that I propose drives the recurrent emergence of the mutation, as in the context of carbapenem therapy, KP survival occurs. Whilst this mutation may emerge during carbapenem therapy of invasive infection I suggest it is more likely to occur in the gastrointestinal tract in colonising KP strains. The administration of broad spectrum antibiotics to treat invasive infections is known to have large impacts on the gut microbiome and this bystander effect may result in KP adapting to this exposure²¹². Our wild-type strain results in severe infection following minimal doses and it would be ethically challenging to repeat infections, with meropenem treatment, to search for *de novo* 25c>t mutants,

as the number of animals required would be high. This final experiment also highlighted why OmpK36 L3 GD insertions are such a common evolutionary mechanism employed to increase the carbapenem MIC. Infections with these strains are robust compared to those without L3 GD insertions and infection proceeds unhindered despite meropenem administration.

I did not identify any other synonymous *ompK36* mutations in this collection or identify any other regions undergoing high selective change. I therefore propose that truncations, reduced expression by transcriptional modification or by the 25c>t mutation and L3 insertions constitute the mechanisms by which KP increases its resistance in the face of carbapenem pressure in clinical environments. However, whilst the focus of this work was a single gene, it paves the way to look at other mutations that may have been considered ‘silent’ in other bacterial genes. Such work should not be limited to KP, but include all bacterial species, and include genes involved in antimicrobial resistance as well as other physiological processes involved in virulence and beyond.

6. Conclusions and Future Perspectives

Small animal models have been used widely in preclinical research and development to explore the potential of novel therapeutic strategies. Although there are issues of consistency and appropriateness of approach, this strategy is likely to continue. This work has not been limited to infectious diseases, however, even in advance of the COVID-19 pandemic, international and national level funding was being channelled into antimicrobial research. Whilst the antimicrobial pipeline of new classes of agents is running somewhat dry there remain many questions regarding the molecular mechanisms leading to resistance to current drugs; the answers to such questions may have real relevance to future drug design. In this thesis, I explored a contemporary threat, CRKP, and worked towards building a more coherent understanding of the role of OmpK36 mutations in the resistant phenotype (Summarised in **Figure 6.1**). The experimental methods used here aimed not only to provide precise molecular mechanisms but also to use *in vivo* infections to assess the impact these mutations have during mammalian infection. In combining these approaches, I have gained insight into the role of OmpK36 in both carbapenem resistance and virulence, which both converge functionally on this central non-selective KP outer membrane porin.



The diminutive size of mice and the ethics of using large cohorts of animals pose challenges to researchers in the field. In this thesis I developed a novel ventilator associated female BALB/c KP pneumonia model and validate a fibre-optic guided inoculation approach. Whilst this is technically challenging it provides a mode of infection akin to that observed in critically unwell patients. Beyond this, I incrementally added outcome variables to assess the host response, with the aim of strategically assessing humoral factors associated with sepsis and septic shock severity in humans. Technological advancements that allow multiple cytokines and chemokines to be measured from small serum volumes have made this feasible. In adding myeloid cell quantification from the lungs this model now provides a high-fidelity multi analyte platform to assess both the host and bacterial response to both changes in KP genetics (e.g. *ompK36* mutations) or outcomes following treatment (e.g. meropenem administration). Over the course of this work several novel non-antibiotic approaches to KP therapy have been published including therapeutic monoclonal (mAb) treatments. These include the use of anti-O1, O2 LPS^{36,213} and anti-mrA (type 3 fimbrial subunit)²¹⁴ targeting mAbs. These molecules were tested in proof-of-principle *in vivo* models by intranasal KP administration and survival (with lung pulmonary burdens in one case) assessed as primary outcome variables. All demonstrated promising efficacy and it would be of significant interest to test these treatments in our model. This could extend our understanding of which aspects of the host response are modified and translate into improved survival.

I highlighted a lack of physiological assessments in murine KP pneumonia models, which I was unable to satisfactorily assess in our model. There is a paucity of available equipment to measure heart rate, respiratory rate, body temperature and oxygen saturations in mice. When these measures have been assessed, it has been carried out on individual animals and for it to be feasibly employed in our model a multiplex approach would be required. This would allow data acquisition to take place simultaneously in cohorts of animals. Surgically implanted sensor systems, such as those manufactured by DSI™ (<https://www.datasci.com/>), may provide such a solution but have not been applied to bacterial pneumonia models in mice. It is interesting to compare this to data collected from critically unwell patients, and the MIMIC-III database provides a useful contemporary dataset for comparison. Here, data from 53,423 distinct adult and 7870 neonatal critical care admissions are associated with metadata including physiological measurements and laboratory tests. A mean of 4579 charted observations (which include heart rate, respiratory rate, body temperature and oxygen saturations) are available for each patient, yet in murine models these variables are barely reported. In the same dataset a mean of only 380 laboratory results per hospital episode are available, including measurements equivalent to serum cytokines in mouse models. It raises an important question as to if our laboratory models are skewed towards what we *can* measure,

rather than what we *should* be measuring, limiting the translatability of our studies. In future work, I would like to develop these aspects of the model to improve the characterisation of preclinical therapeutic candidates into disease modifying agents in patients.

I adopted a reductionist approach and focussed on a single mouse breed, single mouse sex and single KP strain for pragmatic reasons. There was a solid evidence base for selecting this combination, based on my literature review, and both economic and time constraints prevented testing the effect of systematically altering these factors. This approach was validated by my *in vivo* results, as the phylogenetic and onward transmission data associated with *OmpK36* L3 insertions and the 25c>t mutation in clinical isolates, matched those predicted by my mouse infections. Nonetheless, there is a significant genetic heterogeneity in KP^{20,187} and optimising infections with alternative KP isolates, especially those belonging to high risk STs, may provide better models for evaluating future therapeutics. To that end, I have obtained a clinical ST258 isolate that is able to infect immunocompetent mice¹⁵¹ and will evaluate this in future work to establish infections in our model setup. In my view, this improvement in the model could generate a valuable platform for assessing vaccine candidates, which, for multidrug resistant nosocomial infections are notably lacking²¹⁵. This work only covers KP, however this model could be adapted and optimised for infections with the other WHO critical priority organisms, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Work on all these pathogens is pressing as mounting resistance carries the threat of a 'post-antibiotic era' for some organisms.

In much of this thesis, I focus on the biology of *OmpK36*, an OM porin that lies at the intersection between achieving nutrient diffusion in microbial physiology and antibiotic diffusion in our clinical treatment of KP disease. Whilst we have extensively explored the molecular mechanisms underpinning *OmpK36*-mediated resistance and their impact on *in vivo* fitness this relates to a single class of antibiotics. Other key classes of drugs that are used in the treatment of KP are fluoroquinolones and aminoglycosides. Future work should explore the impact that resistance mutations to these agents have on fitness. In particular, chromosomal mutations in the DNA gyrase subunits, *GyrA* and *ParC*, confer fluoroquinolone resistance in KP, yet their impact on fitness has not been evaluated in animal models²². It is conceivable that these mutations may impact on topoisomerase activity and result in KP attenuation. Whilst most aminoglycoside resistance determinants functionally inactivate these drugs, for example by acetylation in the case of *aac(3)-Ia*²¹⁶, a new threat is pan-aminoglycoside resistance that can be achieved by 16S RNA-methyl transferases (16SRMTases). Mechanistically, these enzymes, which are found increasingly in the KP resistome²¹⁷, modify the 16S aminoglycoside ribosome binding sites and preclude their activity. The impact that this modification has on the efficiency of protein synthesis is unknown and therefore, it could

be hypothesized to reduce virulence *in vivo*. In assessing the fitness cost of these mutations, which may be neutral, we may be able to predict the future epidemiology and spread of such antibiotic resistant determinants, as were able to do for OmpK36 mutations.

I extensively evaluate two sets of mutations that occur in OmpK36, namely the regulatory 25c>t mutant and L3 GD insertion pore constricting mutation. These mutations occur in the N-terminal coding region and centre of the open reading frame, respectively. Truncations can occur throughout the *ompK36* gene; however, I show these to be present at low frequency in clinical isolates. OmpK36 insertion into the OM requires a series of sequential steps; transcription and translation, Sec-dependent transport to the inner membrane, followed by maturation, folding and trans periplasmic transport to the OM. The latter process is facilitated by the β -barrel assembly machine (BAM complex) which requires a C-terminal β -signal motif for recognition²¹⁸ (**Figure 6.1**). Thus far, mutations in the *ompK36* β -signal motif or mutations in the BAM complex subunits have not been evaluated and may represent alternative pathways by which KP can reduce OM OmpK36 abundance thereby increasing resistance. In our characterisation of the 25c>t mutation, that occurs in the N-terminal Sec-dependent signal sequence, I did not identify any non-synonymous mutations, suggesting no role for such mutations in generating a resistant phenotype. If C-terminal mutations do occur in adaptation and the development of resistance these could then be included the bioinformatic tools used to interrogate whole genome sequencing data, such as Kleborate²², which already includes L3 insertions after our publication⁶⁶.

The *ompK36* 25c>t mutation demonstrates, for the first time, a resistance mutation mediated by an RNA secondary structure. The synonymous nature of the 25c>t may have led to it being overlooked in previous genomic analyses by others working in the field. Its recurrent emergence, independently, in clinical KP isolates is an elegant example of convergent evolution in achieving a beneficial antibiotic resistant phenotype. The identification of the 24c>t, always in combination with a 25c>t, adds an additional layer of complexity in which the selective pressure to enhance OmpK36 expression was greater than reducing its OM abundance. Of note, this only occurred in OmpK36 variants which had L3 insertions which would intrinsically enhance carbapenem resistance without requiring OM depletion. Given I demonstrate that L3 insertions have little *in vivo* fitness cost this combination of events is evolutionarily sound. In combining all the data into a single working model, I propose three routes to OmpK36 mediated resistance. These are, in order of decreasing fitness costs; OmpK36 truncations, 25c>t mediated OmpK36 depletion and L3 insertion mediated OmpK36 pore constriction. In OmpK36 pore constriction, OM depletion by a 25c>t mutation, is no longer beneficial as the very high carbapenem MICs achieved through pore constriction negate any additional benefit of increasing MICs through reducing OmpK36 abundance.

The underlying mechanism driving 25c>t mediated OmpK36 OM depletion leads to broad questions regarding bacterial gene expression including whether similar mutations occur in other genes that may confer antibiotic resistance or regulate other bacterial processes. Alternatively, whilst the mutation is inhibitory there may be cases where N-terminal mutations may enhance gene expression but have been ignored, or not evaluated, due to their synonymous nature. The task of identifying such mutations would be daunting but could include identifying mutations in large genome datasets, then predicting RNA structures with computational algorithms. Whilst such RNA folding tools exist, without additional laboratory-based methods, such as DMS-MaPseq that we employed²⁰⁶, RNA structures would remain a prediction. In turn, we show dramatic changes in protein abundance, but this would need to be assessed by *in vitro* studies on a mutation-by-mutation basis. In combining genomics with molecular genetics and translational *in vivo* modelling we were able to determine the emergence, spread and fitness implications of this mutation. In the case of OmpK36, its key role in carbapenem resistance, lead us to pursue this, however, in genes of unknown function it may be more difficult to make a case for such extensive investigation.

In the first study of its kind, using a *post-hoc* analysis of a large, international, multicentre cohort trial of CRKP infections Wang et al. attempted to associate KP bacterial genetics with patient outcomes²¹⁹. However, no independent associations were identified between the infecting KP ST, capsule type or siderophore systems with 30-day mortality. In addition, they did explore *ompK35* and *ompK36* mutations, and found no significant impact on mortality, however all mutations in either, or both genes, were included into a single category. Data contained in this thesis would suggest a more detailed analysis is warranted. Indeed, I show that *ompK35* truncations come with minimal fitness cost, whereas the loss of *ompK36* significantly attenuates our laboratory strain *in vivo*. Detailed further analysis may also allow us to understand the fundamental role that widespread OmpK36 L3 insertions play in modifying the response to treatment in patients.

Finally, and concluding this thesis, I would like to turn to new drugs that have recently become available in the treatment of CRKP. New combination therapies, in which a carbapenem or third-generation cephalosporin are combined with new β -lactamase inhibitors (which inhibit carbapenemases), are now in clinical use. These now represent true last line agents in our armamentarium and include meropenem/vaborbactam (MER/VAB), ceftazidime/avibactam (CAZ/AVI) and imipenem/relebactam (IMP/REL). The combination of aztreonam/avibactam (AZT/AVI) is also being evaluated for the treatment of NDM-producing CRKP. While relatively rare overall, cases of resistance emerging to the novel combination therapies (MER/VAB, CAZ/AVI, IMP/REL, AZT/AVI) during therapy have been reported²²⁰. Resistance

typically involves mutations or increased expression of a beta-lactamase (including KPC and AmpC enzymes) ²²¹. However, loss or downregulation of OmpK36 has already been associated with increased resistance to MER/VAB ¹⁹⁵, CAZ/AVI ^{222–224} and IMP/REL ^{223,225}. Future work, already in progress, is evaluating the impact of our OmpK36 resistance mutations on these drug combinations, utilising our isogenic strain collection. Additionally, as new mutations are discovered or as the results of contemporaneous prospective KP collections are released (e.g. future EuSCAPE studies) we can use this system to evaluate mutations and understand functionally how they circumvent these newly licensed agents.

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8.1 Appendix 1

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